In-vitro Induction of Embryonic Stem Cells into Neural Lineage through Stromal Cell-Derived Inducing Activity

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ABSTRACT

Background: Embryonic stem (ES) cells are pluripotent and can differentiate into stem and progenitor cells of the three primary germ layers of the embryo—endoderm, mesoderm, and ectoderm. Stem cell technology could induce and differentiate ES cells into cells of interest *in-vitro*, but with different efficacies in terms of yield, purity, viability and functionality. The derived cell products can potentially serve as a novel regimen for survivors with permanent neurological deficits.

Objectives: The induction of ES cells into neural cell lineage is currently facing the hurdles of low yield and impure population. A novel induction method is the prerequisite for providing a sufficiently pure neural cell population for cell replacement in neurodegenerative diseases. The applicability of the stromal cell-derived inducing activity (SDIA) of ancillary cells, mimicking the *in-vivo* micro-environment of the central nervous system (CNS), in the induction of ES cells into neural cell lineage was elucidated *in-vitro* and *in-vivo*.

Materials & Methods: Mouse ES cell lines, D3 and E14TG2a were propagated in either serum-based or serum-free culture media with or without the support of either mouse primary embryonic fibroblasts (PMEF) or mouse fibroblast cell line STO on different plastic culture wares. The optimal condition was pursued for downstream studies. The SDIA of mouse neural precursor C17.2 cells, areola-derived L cells and Wnt-3A-secreting L-Wnt-3A cells in the induction of mouse ES cell lines, D3 and E14TG2a, were studied *in vitro* by non-contact co-cultures and conditioned medium

(CM)-supplemented cultures. Stromal cells were analyzed for neurotrophic and neuro-protective factors. ES cell-derived cell products were immunologically and molecularly characterized for neural cell markers and lineage commitment, respectively. *In-vivo* studies of the SDIA-induced cell products were conducted in the mouse model of brain ischaemia induced by transient bilateral common carotid artery occlusion (BCCAO) and reperfusion. Behavioural assessment of ischaemic mice after intracranial implantation of ES cell-derived cell product onto the striatum was performed in the water maze system to evaluate spatial learning and memory ability. The tracking of bromo-deoxyuridine (BrdU) labelled ES cell-derived cell products was made on brain sections of transplanted mice and teratoma formation was also examined.

Results: ES cells were best maintained and passaged *in-vitro* on PMEF in culture medium supplemented with defined serum as revealed by the preponderance of the stage-specific embryonic antigen-1 (SSEA-1).

Brain derived neurotrophic factor (BDNF), glial cell line derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), neurotrophin-3 (NT-3), insulin-like growth factors-1 and 2 (IGF-1 and -2), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and erythropoietin (EPO) were detected in C17.2 cells, L cells and L-Wnt-3A cells suggesting the neurotrophic and neuro-protective potential. The immuno-reactivity of neuroepithelial stem cell intermediate filament (nestin), beta-tubulin class III (TuJ III), microtubule-associated protein-2 (MAP-2), tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP),

and myelin binding protein (MBP) in the ES cell-derived cell products suggested the growth of a cell population in hierarchy of the neural cell lineage. The expression of neural cell lineage-related genes, *Pax6*, *Otx1* and *Nurr1*, indicated neuroectodermal precursors, in addition to neural stem cells, mature neurons, dopaminergic (DA) neurons, astrocytes and oligodendrocytes, in ES cell-derived cell products. The products were likely free from mesodermal and endodermal cells as no gene products of *Brachyury* and *α-fetoprotein (AFP)* were amplified, respectively. Nevertheless, weak expression of the transcription factor Oct-4 was noted. The SDIA was remarkable in both co-cultures and CM-supplemented cultures, despite the readouts derived from half-diluted CM was a bit lesser. A proportion of 75% nestin$^+$ colonies were achieved in the co-cultures of D3 and C17.2.

In-vivo studies of the SDIA-induced cell products displayed a significant improvement in spatial learning and memory ability in ischaemic mice post-transplant as compared to ischaemic mice receiving sham operation. Localization of BrdU-labelled cells along the needle track of injection in the brain was evident. There was migration of BrdU$^+$ cells in the vicinity and few BrdU$^+$ cells were examined in brain tissues contra-lateral to the site of injection. Nevertheless, teratoma developed in a mouse.

Conclusion: A hierarchical population of neural cell lineage could be *in-vitro* derived from ES cells through the SDIA of neural precursor cell line C17.2. The clinical relevance was evident in a mouse model of brain ischaemia. However, there is still

room for improvement to prevent the formation of post-transplant teratoma. The finding of this study may help translating to human setting.

ABSTRACT [IN CHINESE]

綜述：

背景：

胚胎幹細胞具有多潛能分化性，可以分化成內中外三個胚層的幹細胞及祖細胞。幹細胞技術可以在體外誘導分化胚胎幹細胞到所需的靶細胞，但其過程複雜，產量、純度、成活力及功能都不穩定。誘導後細胞爲中風後病人長期神經遺留症狀的缺損提供了一種新的治療辦法。

實驗目的： 在體外採用具有誘導活性基質細胞作爲滋養層細胞，模仿體內的神經系統微環境，研究其對於胚胎幹細胞的神經方向誘導作用及所誘導細胞在體內的作用。

材料和方法：採用了有/無血清培養基，有/無鼠原代胚胎成纖維細胞或鼠成纖維細胞系 STO 滋養以及不同材料的塑膠皿等方法來培養鼠胚胎幹細胞系 D3 和 E14TG2a，並尋找最佳的適應環境。我們分別用不接觸共同培養法和條件培養基法來研究鼠神經祖細胞 C17.2、 小腔來源的 L 細胞 ，Wnt-3A-分泌型 L-Wnt-3A 細胞對鼠胚胎幹細胞系 D3 和 E14TG2a 的誘導活性。我們用分子生物學方法檢測基質細胞神經營養因數和神經保護因數的表達；用免疫組化和分子生物學方法檢測胚胎幹細胞分化產物標誌物的表達。體內實驗，我們採用暫時夾閉雙側頸總動脈的方法造成小鼠腦缺血及缺血後再灌注，將基質細胞誘導的產物移植到缺血小鼠的腦紋狀體部位，用水迷宮來檢測移植後小鼠的空間學習及記憶能力，並在移植小鼠的腦片上檢測 BrdU 標記的胚胎幹細胞來源的細胞產物以及畸胎瘤形成。

胚胎幹細胞可以良好的生存於體外以原代成纖維細胞及一定血清濃度的培養基中，並在一定的時期表現胚胎時期的特定的標誌物：SSEA-1。我們用分子生物的方法檢測到 C17.2 細胞，L 細胞，和 L-Wnt-3A 細胞有 *BDNF, GDNF, CNTF, NGF, NT-3, IGF-1, IGF-2, bFGF, VEGF* 和 *EPO* 的存在，說明這些細胞有神經營養和神

經保護的作用。我們檢測到胚胎幹細胞分化產物具有 nestin , TuJ III, MAP-2, TH, GFAP 和 MBP 免疫陽性反應，說明他們可以向神經細胞系分化。

神經細胞系相關基因表達 *Pax6, Otx1* 和 *Nurr1* 表明胚胎幹細胞的誘導產物中除了神經幹細胞、成熟神經元、多巴胺神經元、膠質細胞和少突膠質細胞外，還有神經外胚層祖細胞誘導產物中沒有檢測到中胚層以及內胚層基因 *Brachyury* 和 *α-fetoprotein*。轉錄因數 *Oct-4* 呈現弱陽性反應。儘管在一半稀釋的條件培養液中基質細胞誘導活性有所下調，但在共同培養和條件培養液中都表現出增強。和 D3 以及 C17.2 細胞共同培養的胚胎幹細胞誘導產物中 75% 的克隆 nestin 陽性。體內試驗表明基質細胞活性誘導的細胞能夠明顯的提高腦缺血的小鼠學習及記憶能力。BrdU 陽性的細胞主要局限於注射部位，有一些遷移到鄰近的部位，也有一些遷移到注射部位的對側。然而，其中一頭老鼠的注射部位有畸胎瘤長出。

結論：胚胎幹細胞可以被神經祖細胞系 C17.2 作爲滋養層細胞而誘導成不同的神經系細胞。缺血鼠模型的檢測證明其具有臨床的應用潛能。然而，對於移植後畸胎瘤的出現還需要進一步的改善。另外具體的臨床應用機制還需要進一步闡明。該實驗發現將有助於用於人體。

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AA	Amino Acid
AFP	α-Fetoprotein
APES	Aminopropyl triethoxy-silane
Bp	Base pair
BCCAO	Bilateral Common Carotid Artery Occlusion
BDNF	Brain Derived Neurotrophic Factor
bFGF	Basic Fibroblast Growth Factor
BMP	Bone morphogenetic protein
BrdU	5-Bromo-2'-deoxyuridine
cDNA	Complementary DNA
CM	Conditioned Medium
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
CXCL-12	Chemokine (C-X-C motif) ligand 12
DA neuron	Dopaminergic neuron
DAPI	4',6'-diamidino-2-phenylindole hydrochloride
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
DPBS	Dulbecco's Phosphate-Buffered Saline
dpc	days post coitus
EB	Embryoid Bodies
EC	Embryonic carcinoma cell
EDTA	Ethylenediaminetetraacetic Acid
EG	Embryonic germ cells
EPO	Erythropoietin
ES cell	Embryonic Stem cell
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate

FL	Flt-3 Ligand
Fn	Fibronectin
GABA	Gamma-aminobutyric acid
GAPDH	Glyseraldehyde-3-phosphate Dehydrogenase
G-CSF	Granulocyte Colony Stimulating Factor
GDNF	Glial cell line Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GS	Goat Serum
H & E	Haematoxylin and Eosin
HSC	Haematopoietic stem cells
IGF-1	Insulin Growh Factor-1
IGF-2	Insulin Growh Factor-2
IS	Ischaemic stroke
IS+C	Ischaemic mice with grafted cells
ITS	Insulin-Transferrin-Sodium Selenite media
ITSFn	ITS supplemented with Fibronectin
KO DMEM	Knock-Out Dulbecco's Modified Eagle's Medium
LIF	Leukemia Inhibitory Factor
MAP-2	Microtubule Acidic Protein-2
MBP	Myelin Binding Protein
MSC	Mesenchymal Stem Cells
Nestin	Neuroepithelial stem cell intermediate filament
NGF	Nerve Growth Factor
NSC	Neural Stem Cells
NT-3	Neurotrophin-3
Oct-4	Octamer binding transcription factor
OD	Optical Density
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
PI	Popidium Iodide
PMEF	Primary Mouse Embryonic Fibroblasts

RA	Retinoic Acid
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCF	Stem Cell Factor
SDIA	Stromal cell-Derived Inducing Activity
SHH	Sonic Hedgohog
SR	Serum Replacer
SSEA-1	Stage-Specific Embryonic Antigen-1
TAE	Tris-Acetic acid-EDTA Buffer Solution
TGF	Transforming Growth Factor
TH	Tyrosine Hydrolzase
TPO	Thrombopoietin
Tra1-81	Tumor rejection antigen 1-81
TRITC	Tetramethylrhodamine Isothiocyanate
TuJ III	Class III beta Tubulin
VEGF	Vascular Endothelial Growth Factor
Wnt-3A	Wingless-related MMTV integration site 3A

Chapter 1 Introduction

1.1 Embryonic stem (ES) cells

ES cells originate from the blastocyst stage of the embryo prior to implantation onto the uterine wall. In mouse, the pre-implantation embryo is made up of a constellation of cells with an outer layer of trophectoderm enclosing a fluid-filled cavity of blastocoel and a cluster of cells known as the inner cell mass (ICM). ES cells are derived from the ICM (Evans et al. 1981; Martin 1981). They lack the G1 checkpoint and are almost in the S phase of the cell cycle, during which they synthesize deoxyribonucleic acid (DNA). Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication. They express the transcription factor, Octamer-binding transcription factor-4 (*Oct-4*; (Okamoto et al. 1990) and are capable of undergoing an unlimited number of symmetrical divisions without differentiating to give rise to a colony of identical cells or clones that have the same properties as the original cell. Proliferating ES cells maintain stable normal diploid karyotypes (Odorico et al. 2001). However they show no sign of X-chromosome inactivation, which takes place normally in all female somatic cells that one of the two X-chromosomes becomes permanently inactivated (Marshak 2001; Smith 2001).

ES cells are pluripotent and can differentiate into stem and progenitor cells of the three primary germ layers of the embryo—endoderm, mesoderm, and ectoderm (Wiles 1993; Smith 2001). ES cells are also capable of colonizing the germ line and giving rise

to egg or sperm cells (Hubner et al. 2003; Geijsen et al. 2004). By injecting ES cells into the cavity of another blastocyst and transferring the engineered embryos into the uterus of a pseudo-pregnant female mouse, chimeras were formed resulting to progenies derived from both donor ES cells and the recipient blastocyst (Beddington et al. 1989). When ES cells were implanted under the skin or into the kidney capsule of adult nude mice, tumors in the form of teratoma, which is characteristic of the outgrowth of cells of the three dermal layers (Ringden et al. 2003). As ES cells are cultured *in-vitro*, they can differentiate spontaneously by forming embryoid bodies (EB; Martin et al. 1975; Itskovitz-Eldor et al. 2000; Toumadje et al. 2003; Heo et al. 2005), which resemble teratomas *in-vivo*. EB resemble early post implantation embryos and consist of a disorganized array of differentiated or partially differentiated cell types of three primary germ layers of the embryo (Reubinoff et al. 2000; Odorico et al. 2001; Gepstein 2002).

Mouse ES cells were isolated for the first time in mice in 1981 (Evans and Kaufman 1981; Martin 1981). It was a major breakthrough in the field of developmental biology. ES cells were allowed to grow indefinitely in an undifferentiated state, without senescence, in the presence of leukemia inhibitory factor (LIF; Williams et al. 1988) and mitotically inactivated primary mouse embryonic fibroblasts (PMEF) or STO mouse fibroblasts (Martin and Evans 1975). Murine ES cell lines are proven to be able to differentiate *in-vitro* into a variety of embryonic and adult cell types of three embryonic germ layers (Table 1). Thereafter many ES cell lines are available from cattle (Cherny et al. 1994), pig (Gerfen RW et al. 1995), primates (Thomson et al. 1995). In 1998, Thomson and his group at the University of Wisconsin-Madison successfully established

ES cell line from human early embryos (Thomson et al. 1998). To date, approximately 30 human ES cell lines are developed (Gearhart 2004).

Table 1 ES cell derivation

Germ Layer	Cell types	References
Endoderm	Pancreatic Islets	(Lumelsky et al. 2001)
	Adipocyte	(Dani et al. 1997)
Mesoderm	Hematopoietic cells	(Wiles et al. 1991)
	Cardiomyocyte	(Doetschman et al. 1985)
		(Maltsev et al. 1993)
Ectoderm	Neuron	(Bain et al. 1995)
	Keratinocyte	(Strubing et al. 1995)
		(Bagutti et al. 1996)

1.2 Stem cell plasticity

Apart from ES cells, there are many types of stem cells namely, haematopoietic stem cells (HSC), neural stem cells (NSC), skin stem cells, mesenchymal stem cells (MSC), pancreatic progenitor and many other site-specific stem cells. They are essentially undifferentiated cells that can be found in the developing embryo, foetus and adult body. All have the capacities of self-renewal and differentiation (O'Shea 2004) to replenish cell loss in a homeostatic manner throughout life (Lindblad 2001). In optimal *in-vitro* culture conditions, they can be trans-differentiated into cells of different lineages (Burke et al. 2005).

1.2.1 Differentiation and trans-differentiation of lineage-restricted stem cells

1.2.1.1 Multilineage differentiation *in-vitro*

Recent publications demonstrated that ES cells could be induced to form the desired cell phenotype *in-vitro* when appropriate culture conditions were provided (Maltsev et al. 1993; Hole 1999; Rosenberger 2003; Schulz et al. 2004). These included cardiomyocytes, hematopoitic cells, neural cells, myocytes, adipocytes, hepatocytes, epithelial cells, liver cells and many others. It was also shown that ES cells could also give rise multipotent lineage-restricted stem cells such as HSC, NSC, liver stem cells and etc (Hole 1999; Chen et al. 2003; Bibel et al. 2004).

1.2.1.2 Trans-differentiation

Under suitable conditions, multipotent tissue-specific stem cells are capable of producing a whole spectrum of cell types, regardless whether progenies are derived from the same germ layer (Passier et al. 2003). HSC could be converted into many other types of cell under suitable condition, including liver and neural cells (Hao et al. 2003). NSC could be converted into hematopoietic cells (Shih et al. 2002), and skeletal muscular cells (Vescovi et al. 2002). Bone marrow stromal cells could be turned into a variety of non-haematopoietic cells of ectodermal, mesodermal and endodermal origins such as myocytes, neural cells and hepatocytes (Tao et al. 2003).

Trans-differentiation is interesting as it raises the possibility of obtaining autologous lineage-restricted healthy stem cells and converting them into cells of interest for tissue repairs without difficulties in collecting lineage-specific stem cells from allogeneic donor. It is now premature to argue that adult somatic stem cells should be pursued in preference to ES cells. In facts, these two areas of research are complementary and synergistic.

1.2.2 Prospective applications of stem cells

1.2.2.1 Basic research on development

ES cells are undoubtedly used as a key research tool for understanding molecular machinery, growth factors, cytokines, chemokines, adhesion molecules, ligands and other yet unknown substances during the process of differentiation from the embryonic stage to the adult phenotype. They may help elucidating the causes of birth defects so that prospective measures can be implemented for disease correction and prevention (Evans 1989; Rathjen et al. 1998). Besides, chromosomal abnormalities in early development can also be explored with ES cells in which the developments of early childhood malignancies are embryonic in origin.

1.2.2.2 Study of human disease

ES cells provide a good model for the study of human-only diseases including acquired immuno-deficiency syndrome and hepatitis C, which are severely constrained by a lack of *in-vitro* models.

1.2.2.3 Cancer research

ES cells can nourish an unlimited supply of lineage-specific stem cells catering for the physiological need. There is a plethora of evidence suggesting the attributes of

cancer stem cells in human malignancy. ES cells can be a good vehicle for delivering genes to specific tissues in the body and restoration of the immune function to tackle cancer. Currently many ways have been devised to utilize specialized cells derived from ES cells to target specific cancerous cells.

1.2.2.4 Drug screening

Despite *in-vivo* testing in animals is a mainstay of pharmaceutical research, the efficacy in human can hardly be foreseen. Human ES cells can derive a stable and pure population of cells of interest for drug testing in a real, live and fairly normal manner prior to clinical trials (McNeish 2004).

1.2.2.5 Cell therapy

Degenerative disorders like myocardial infarction, Parkinson's disease, diabetes are attributable to the progressive, either acute or chronic, loss of functional cells in disease, injury and/or aging. The replacement of the worn-out or injured cells by quality cells to restore the normal physiological functions of tissues or organs is the underlying principle of regenerative medicine (Rathjen et al. 1998; Fodor 2003; Doss et al. 2004).

Despite recent advances in transplantation medicine, there is a shortage of donor organs. However, ES cells could potentially revolutionize medicine by providing an unlimited and renewable supply of cells capable of replacing or repairing tissues that

have been damaged in degenerative disorders or diseases leading to the deprivation of functional cells. ES cell-based replacement therapy was reported to be efficient (Rohwedel et al. 1994; Delcarpio et al. 1995; Barberi et al. 2003).

1.3 Neuro-degenerative diseases and cell therapy

1.3.1 Neuro-degenerative diseases

There are mainly two types of cells: neurons and glial cells in the brain. Neurons transmit electric signals, controlling all actions of body parts. Glial cells are categorized in oligodendroglia and astroglia. Oligodendrocytes are responsible for producing myelin, a fatty substance that provides electrical insulation on the axons. Death of oligodendrocytes results in demyelination, halting communication between the brain and the rest of the body. Astrocytes break down and remove harmful proteins, as well as secrete proteins called neurotrophic factors, which help neurons survive and grow. They also respond to injury. They clear away debris, which results in formation of glial scarring (Siegel 1999).

Both neurons and glial cells are important. Disorder in one of them in neurodegenerative disease would probably cause dysfunction of brain, and seriously affects the daily life of patients. Moreover, there is usually no effective therapy for neurodegenerative disease.

With the establishment of ES cells and their two characteristics, it provides a big hope for supplying unlimited source, by simply inducing their differentiation into certain cells of interests, to replace the cells loss and dysfunction cells in neurodegenerative diseases, include the stroke (Famularo et al. 1998; Nadareishvili et al. 1999), spinal cord injury (Baleriaux 1991), Parkinson's disease (Hirsch et al. 1988; Siegel 1999), Alzheimer disease (Arenas 2002), multiple sclerosis (Foote et al. 2005).

1.3.2 Neuro-regeneration

Over the past decades, convincing evidence suggested that neurogenesis in the adult CNS is a continuous physiological process. The production of new neurons is noted in the sub-ventricular zone and the sub-granular zone of the dentate gyrus (Galli et al. 2003). It was demonstrated in animal models of disease of or injury to CNS, such as seizures and traumatic brain injury, neurogenesis would be induced. Global forebrain ischaemia and focal ischaemia in adult mammals were noted to enhance neurogenesis in the dentate gyrus (Nakatomi et al. 2002; Hallbergson et al. 2003; Briones et al. 2005). Besides, it was reported that newly formed neurons can replace degenerated neurons at the lesioned site and improve the neural function. However, the mortality of the new neurons is high and over 80% died within the first six weeks. Approximately 0.2% of the injured cells could be replaced by newly generated neurons (Arvidsson et al. 2002).

Besides, it was reported that neural stem cells in brain constitutively secrete neurotrophic factors, neural inducing factors, survival promoting factors that promote extensive neuronal growth after injury (Lu et al. 2003; Serpe et al. 2005).

1.3.3 Cell sources for neuro-regenerative therapy

1.3.3.1 Comparison of stem cells

There are mainly two categories of stem cells: embryonic and adult stem cells. Recently, NSC, HSC and MSC are the adult stem cells that widely used in differentiation research (NIH 2001). Table 2 shows the comparison of ES cells and various adult somatic stem cells.

In fact, embryonic and adult stem cells vary significantly in terms of potential use in cell-based therapies. They show differences in the number and type of differentiated cells which they can derive. ES cells are pluripotent that become all cell types of the body. Adult stem cells, especially NSC are generally limited to differentiating into different cell types of their tissue of origin. This may be an advantage to yield a relatively homogeneous cell population having a high purity, while ES cells usually yield heterogeneous cell products with a low purity.

ES cells in large numbers can proliferate indefinitely in culture, while adult stem cells are rare in mature tissues and could only grow in cultures for a shorter time than that of ES cells. Adult stem cells are not easily harvested from living people, especially the NSC. There is little adult stem cell line established at the moment. That is the reason why many works were done on ES cells than adult stem cells.

A potential advantage of using adult stem cells is that the risk of immune response is highly reduced as the graft is originated from the same patients. This is a significant advantage as immune rejection is a difficult problem that can only be circumvented with immunosuppressive drugs. On the other hand, immune response to ES cells may be elicited although it has not been determined in human experiments (Drukker et al. 2004; Li et al. 2004). Moreover, application of ES cells in cellular therapy poses the risk of teratomas formation (Reubinoff et al. 2000; Arnhold et al. 2004).

1.3.3.2 Stem cells in neuro-regenerative therapy

The main rationale of cellular therapies for neuro-regeneration is to replace degenerated or lost neurons and glial cells in the affected region, to re-establish and to maintain the functional neuronal circuitry in the brain (Dinsmore 1998). Furthermore, cell-based approaches could also exert neurotrophic efforts to tissue surrounding the injured area and help promote survival, migration and differentiation of endogenous stem and precursor cells by secreting survival promoting factors, migratory factors or neural inducing factors (Alzheimer et al. 2002; Lazarini et al. 2003; Yoshimura et al. 2003; Tran et al. 2004; Cheng et al. 2005).

Cellular therapy employing large numbers of *ex-vivo* derived viable neural cells may hold promise to patients with neuro-degenerative disease by replenishing the loss of functional neural cells. A reliable and accessible cell source is requisite. There are

many potential sources of stem cells which can be utilized to pursue the goal (Passier and Mummery 2003). Among them, ES cells and adult somatic stem cells derived from the CNS and haematopoietic system are very attractive for neuro-regeneration.

Transplantation of human NSC as allograft is currently one of the feasible way used to treat Parkinson's disease. However, the source of adult and foetal NSC is rare and limited. It was reported that the number of foetal NSC isolated from six to ten aborted foetuses could only cater for transplantation of a patient with Parkinson's disease (Hynes et al. 2000). Besides, the human NSC are not easily harvestable for transplantation unless collected during necropsy (Kuhn et al. 2001; Sanai et al. 2004). The isolated adult or foetal NSC are difficult to maintain *in-vitro*. They could be passaged merely a few times. The technical hurdle in the *ex-vivo* expansion of NSC has restricted the generation of sufficient numbers of specialized cells for transplantation.

Therefore, the accessibility of collection of HSC from bone marrow, umbilical cord blood and peripheral blood is advantageous over the harvest of NSC. Trans-differentiation of HSC into neural lineage was reported (Haas et al. 2005) and many pre-clinical studies were conducted in animal models to investigate the regenerative capacity of HSC following brain damage of ischaemic stroke (Hao et al. 2003), by either mobilization of intrinsic HSC pools or transplantation of exogenous HSC. It was interesting to note a three-fold increase of circulating HSC, in terms of $CD34^+$ cell count, in patients with acute cerebral ischaemia (Peterson 2004; Ladeby et al. 2005). A recent pre-clinical study reported functional improvement in rats with focal cerebral ischaemia

after subcutaneous administration of granulocyte colony stimulating factor (G-CSF), suggesting the neuro-protective effect of G-CSF-mobilized HSC (Shyu et al. 2004). In animal studies, the intravenous administration of human umbilical cord blood-derived $CD34^+$ cells to mice and rats promoted neo-vascularization and neurogenesis with concomitant functional improvement (Taguchi et al. 2004).

Preliminary implantation studies in animal models showed that ES cell-derived cell products were functionally active, and integrated into the brain and corrected the phenotype of neuro-degenerative disease (Takagi et al. 2005; Xu et al. 2005). It was noted that the immunogenicity of neural tissue was relatively low as compared with other tissues. This makes ES cell-based cell replacement therapy for neuro-degenerative disease and neurological disorders less likely of immune-rejection.

Compared with ES cells, adult somatic stem cells would raise less ethical concerns (Ringden et al. 2003). However, adult somatic stem cells are not as good as ES cells in term of differentiation potential. Embryonic carcinoma (EC) cells and embryonic germ (EG) cells may be an alternative (Shamblott et al. 1998; Laslett et al. 2003; Przyborski et al. 2004). EG cells are derived from primordial germ-line cells in gonadal ridge at five to nine weeks of development, whereas EC cells originated from teratocarcinoma, usually a tumour of testis. Unlike ES cells, studies EC or EG cells in animals are limited.

The purity of ES cell-derived neural cultures has been problematic. Besides, the implantation to human brain is hindered by the fear of spontaneous formation of teratoma. However, ES cells display many advantages over adult somatic stem cells. They have relatively high differentiation capacity and can readily differentiate into cells of all three germ layers. On the contrary, adult somatic stem cells commit to cells of specific lineage. To date, with the exception of HSC, which display the ability of trans-differentiation, there is no data suggesting that adult somatic stem cells could herald similar differentiation potential of ES cell

Table 2 Comparison of ES cells and adult stem cells (NSC, HSC and MSC)

	ES Cells	Adult Stem Cells		
		NSC	HSC	MSC
Source	Cell Lines	Aborted Foetuses	Bone Marrow Umbilical cord blood	Bone Marrow Stoma
Availability	Unlimited	Limited	Plentiful	Readily
Plasticity	Pluripotent	Multipotent	Multipotent	Multipotent
Product Purity	Heterozygous	Homozygous	Heterozygous	Heterozygous
Product Yield	Moderate	High	Low	Low
Graft-associated Malignancy	Teratoma	Not-reported	Unknown	Unknown

1.4 *In-vitro* derivation into neural lineage

With the withdrawal of LIF, and/or removal from the feeder support and subsequent transferral to a suspension culture, ES cells differentiate spontaneously into multicellular aggregates – EB. With optimized *in-vitro* culture conditions, a high yield of quality neural lineage cells can be derived (O'Shea 2001; Xian et al. 2001).

1.4.1 *In-vitro* induction strategies available

Basically, ES cells resemble early post-implantation embryos (Brook et al. 1997). Cell-cell interactions are critical to normal embryonic development. Simulating these native *in-vivo* interactions in *in-vitro* cultures is a fundamental strategy of ES cell induction. The *in-vivo* conditions can be mimicked by adding chemical stimulants and/or growth factors into the culture medium. A variety of coating matrices are used to enhance adherence to culture wares for better cell growth (Ekblom et al. 1986). Besides, chemically modified culture wares are exploited to promote non-adherent cell aggregates in cultures.

Many protocols were reported to be able to differentiate mouse ES cells into neural cell lineage cells of neurons, astroglia and oligodendroglia. However, the induction efficacy differs in terms of yield, purity, viability and functionality of cells of interest, not to mention the complexity of procedures and processing time required.

1.4.1.1 Chemical agents

1.4.1.1.1 Retinoic acid (RA)

RA is a well known inducing agent to enhance the differentiation of ES cells into specific mature phenotypes. RA is a biologically active form of vitamin A and has been shown to play an important role during embryogenesis (Ross et al. 2000). It is thought to be one of the most important extrinsic inductive agents for neural differentiation *in vitro*. It influences neural development in the early stage of CNS development. In 1982, Jones and co-workers induced P19 teratocarcinoma cell line into neurons and glial cells using RA (Jones-Villeneuve et al. 1982).

P19 cells and ES cells share a number of basic features. Both of them are pluripotent and can differentiate. Bain and co-workers applied a supraphysiological dose of RA to mouse ES cells via EB formation and obtained a high proportion of cells expressing multiple neuronal features (Bain et al. 1995; Fraichard et al. 1995; Strubing et al. 1995; Bain et al. 1996). About 38% of the ES cell-derived product illustrated the neuron-like morphology. They displayed neuronal markers of TuJ-III, neurofilament-M, gamma-aminobutyric acid-synthesizing enzyme and TH. The RA-induced neuron-like cells also demonstrated the electrophysiological property.

However RA is a strong teratogen and is supposed to perturb neural patterning, there are concerns in using RA at concentrations far beyond the physiological range

(Soprano et al. 1995; Sucov et al. 1995; Okada et al. 2004). Besides, it was reported that the absence of RA from the culture medium is required for successful induction of DA neurons (Wilson et al. 2004; Afonso et al. 2005).

1.4.1.1.2 Ascorbic acid

Ascorbic acid is a derivative of vitamin C. It has been successfully showed that ascorbic acid could enrich ES cell-derived TH^+ neurons, together with Sonic Hedgohog (SHH), fibroblast growth factor-8 (FGF-8; Ye et al. 1998; Lee et al. 2000; Briscoe et al. 2001).

1.4.1.2 Growth factors/cytokines

In-vitro uses of cocktails of growth factors and cytokines, including neurotrophins, stimulants and even CM, to mimic the *in-vivo* microenvironment, had been reported to induce ES cells into neural lineage (Schulz et al. 2003). The relative concentrations were empirically and hypothetically determined according to their differential expressions during normal neurogenesis and brain injury. To-date, a number of neurotrophins or stimulants had been identified to be effective in promotion of neural differentiation (Alzheimer and Werner 2002; Hallbergson et al. 2003; Yoshimura et al. 2003; Bondy et al. 2004; Schanzer et al. 2004; Shyu et al. 2004).

As neurogenesis is a very complex process involving many growth factors and cytokines, the supplement of a small cytokine cocktail may not provide an optimal induction condition for ES cells into neural lineage. Further tests on various combinations of factors would be necessary to promote the induction efficacy.

1.4.1.2.1 Neurotrophins

According to Park and co-workers, BDNF and transforming growth factor (TGF)-α could induce a higher number of DA neurons from ES cells. Moreover, GDNF can significantly enhance the differentiation and viability of ES cell-derived DA neurons (Guan et al. 2001; Rolletschek et al. 2001; Park et al. 2004). Barberi and coworkers also demonstrated CNTF could induce astrocytes generation after 18-day serum free condition (Johe et al. 1996; Barberi et al. 2003). NGF was also found to be a potent enhancer of neuronal differentiation, by eliciting extensive outgrowth of processes and expression of neural markers (Schuldiner et al. 2001). NT-3 and NT-4 are the members of NGF family. They increase the number of neurons by increasing neuronal survival, but rather than inducing neuronal phenotype (Johe et al. 1996; Caldwell et al. 2001).

1.4.1.2.2 Stimulants

Basic FGF and EGF are reported to involve in enrichment and expansion of proliferating neural progenitors from ES cells (Reubinoff et al. 2001; Park et al. 2004). VEGF is an angiogenic factor that appeared to play a significant role in the growth and

differentiation of astrocytes in the CNS. Moreover, its up-regulation will induce expression of bFGF. Thus, it suggested that VEGF may indirectly serve in the proliferation of multi-potential neural stem/progenitor cells both in *in-vitro* and *in-vivo* (Schanzer et al. 2004).

Recently, it was shown that there was a cross-talk of hematopoietic factors to neural differentiation. It was suggested that SCF might play a role in cell migration and survival in the developing cortex (Erlandsson et al. 2004). Flt-3 ligand (FL) is a cytokine that promotes the survival, proliferation, and differentiation of hematopoietic progenitors. FL and its ligand have also been identified in brain tumors (Timeus et al. 2001). This further suggests that neuroectodermal and hematopoietic cells share common regulatory pathways. The effects of Erythropoietin (EPO) in glial cell development, especially oligodendrocytes maturation and astrocytes proliferation, were reported by a Japan group (Sugawa et al. 2002). With the treatment of EPO on glial cells, the proliferation of astrocytes and oligodendrocytes were enhanced.

1.4.1.2.3 Signalling molecules

It was reported that ES cells expressed SHH and the corresponding receptors during differentiation. The supplement of SHH to cultures resulted in a significant increase of TH^+ neurons (Lee et al. 2000). Lee and co-workers induced ES cells to form EB in suspension and then stepwise into neural lineage. Similar observation was also noted by Ying and co-workers (Ying et al. 2003).

It was hypothesized that neural fate of ES cells can be acquired through a default mechanism in which inductive signals for alternative fates are eliminated (Tropepe et al. 2001). Bone morphogenetic protein (BMP) is a member of TGF-β superfamily of molecules that strongly inhibit neural differentiation. The binding of signaling molecules, Noggin and Chordin, could promote neural fate specification (Hemmati-Brivanlou et al. 1994).

Wnt signalling has been implicated in the expansion of neural precursor cells in the embryo. Wnt-1 and Wnt-3A of Wnt-protein family cause expansion of neural precursor cells in the developing brain and spinal cord (Derfoul et al. 2004; Muroyama et al. 2004). They are required for the formation of several regions of the CNS, including the midbrain, diencephalon, hippocampus, and dorsal hindbrain (Shibamoto et al. 1998).

The clinical relevance of chemical agents, growth factors, cytokines, stimulants and signaling molecules, either in unity or in combination, in the induction of ES cells into neural cell lineage is still not fully characterized and studied.

1.4.1.3 Culture Selection

1.4.1.3.1 Conditions

Neurons or neural cells are very vulnerable to the culture conditions *in vitro*. Temperature, oxygen/carbon dioxide concentrations have to be carefully maintained for normal growth. Oxygen is toxic to neurons. Anti-oxidants, such as vitamin E, glutathione, pyruvate, catalase and superoxide dismutase, are found to increase survival and growth of neurons. Neuronal survival cultured in 9% oxygen for three days were more than double those in atmospheric oxygen (Brewer et al. 1989). At 0.2% carbon dioxide, neurons would die within hours due to the rapid rise in pH in culture medium (Brewer et al. 1996). Buffered culture medium should be used to avoid the pH changes during the course of experiment.

1.4.1.3.2 Medium

Culture medium plays an important role in neural differentiation. They may help expand and select the neural lineage cells. Basically, there are either serum-based or serum-free media.

Foetal calf serum is an effective supplement for *in-vitro* cell culture because of its complexity and the multiplicity of growth-promoting, cell protection and nutritional factors. However, the favourable effects of serum derived from different batches/lots

vary considerably, not to mention various manufacturers using miscellaneous collecting and producing protocols. Certain factors may be deficient in some batches/lots or present in an excessive levels inhibitory to ES cell growth. It was reported serum inhibits neural differentiation (Pachernik et al. 2005). The applicability of foetal calf sera in ES cell maintenance had to be evaluated.

Serum-free medium emerges as an alternative. Data of recent studies showed reported that N2- and B27-supplemented serum-free media support neural cell growth, but not other lineages (Romijn 1988; Brewer 1995; O'Shea 2001).

Furthermore, adhesion molecule like fibronectin has been supplemented to culture medium (Okabe et al. 1996; Lee et al. 2000). It was reported that fibronectin increases the neural differentiation of ES cells by facilitating the growth of NSC as a monolayer without impairing their differentiation potential and generation of specific differentiated progeny for cell transplantation (Rappa et al. 2004).

1.4.1.4 Transfection of regulator genes using viral vector

Several transcription factors have been demonstrated to regulate differentiation of ES cells into specific cell types. By forced expression of such factors, ES cells are deliberately driven into a specific lineage. ES cells are genetically knocked-in an

antibiotic-resistant gene juxta-positioned to the promoter of a neural transcription factor using a viral vector. During the early stage of differentiation, the expression of antibiotic-resistant gene maintains cell growth of neural lineage in cultures supplemented with the particular antibiotic. Alternatively, the antibiotic blocks the endogenous translation or transcription machineries if alternative progenies do not express the particular enzyme or protein to antagonize the blocking activity. However, immuno-rejection pertaining to the extraneous proteins expressed by the genetically engineered progenies may be a concern in the transplantation setting.

Kim and co-workers demonstrated Nurr1 over-expression in ES cells to induce dopaminergic differentiation *in-vitro* (Chung et al. 2002). Nurr1 is a transcription factor critical for the development of midbrain dopaminergic (DA) neurons. The group showed that Nurr1-induced ES cells expressed DA neuronal markers, such as dopamine transporter, in addition to tyrosine hydroxylase, and gave a four to five-fold increase in the proportion of DA neurons after differentiation. Nurr1-induced ES cells also exhibited depolarization-evoked DA release.

Besides Nurr1, neuroDs (O'Shea 1997) and Sox2 (Li et al. 1998) had also be manipulated to be over-expressed during induction of neural differentiation, and yield neural precursors and neural cells. These studies will serve as a framework of genetic engineering of ES cells by key transcription factor to regulate their cell fate.

1.4.1.5 Stromal cell-derived inducing activity (SDIA)

There are many ancillary cells in the CNS and peripheral nervous system, apart from neurons. They act like feeders to support cell or tissue repair and may even account for other yet unidentified roles in neuro-regeneration.

Kawasaki and co-workers screened various primary cell cultures and cell lines for activities promoting neural differentiation of ES cells in a serum-free co-culture system (Kawasaki et al. 2000). PA6 stromal cell line derived from skull bone marrow was noted to exert SDIA and induce efficiently neuronal differentiation of ES cells in serum-free conditions without the use of either retinoic acid or EB induction. Stromal cell line OP9 derived from mouse calvaria and the embryonic fibroblast line NIH3T3 were also noted to elicit SDIA but to lower extent (Kawasaki et al. 2000; Vieira et al. 2004). The SDIA-induced dopamine-positive neurons were functionally active *in vitro* and demonstrated electrophysiological property. In the *in-vivo* study, the DA neurons could integrate into the striatum of Parkinsonian mice and remained positive for tyrosine hydroxylase expression (Xu et al. 2005). No teratomas formed in the grafted tissue.

Two likely mechanisms were hypothesized to the molecular nature of SDIA. SDIA may mediate neural factors which are either cell surface-anchored factors or labile soluble factors. Alternatively, secreted factors, which are secondarily tethered to the cell surface as exemplified by Wnt and FGF, may be attributed to SDIA. To date, it is still cannot identified all the important uncharacterized substances, including the secretory or

surface-anchored neural factors, and the labile soluble factors. Studies are mainly focusing on screening or selecting stromal cells to exert effective inducing activity on neural differentiation. Nevertheless, SDIA-induced cell product needs more studies to validate its prospective application in cell replacement therapy for neurodegenerative diseases.

Chapter 2 Aims

2.1 Hypothesis and study objectives

Among the different protocols currently available to the induction of ES cells into neural lineage, SDIA should be a more physiologically relevant approach. The lineage-restricted neural progenitor cell line C17.2, which was established from neonatal cerebellum of mouse (Snyder et al. 1992), would best mimic the micro-environment of the CNS and help induce ES cells into neural lineage through SDIA (Lu et al. 2003).

The objectives of this study are: 1) to evaluate the efficacy of neural precursor cell line C17.2 in the induction of ES cells into neural lineage by SDIA and compare the readouts to those derived from L cells, L-Wnt-3A cells and the teratogen RA, and 2) to assess the clinical relevance of the SDIA-mediated cell products in the mouse model of ischaemic brain.

2.1.1 Soliciting an optimal method for ES cell propagation

In-vitro culture conditions of ES cells were elucidated and optimized with respect to 1) different lots and batches of sera available from various suppliers, 2) serum-based versus serum-free culture media, and 3) adherent cultures with and without feeder cell support.

2.1.2 Pursuing alternative SDIA

That neural progenitor cell line C17.2 originated from the cerebellum was hypothesized to carry cues of the microenvironment of mouse brain, which may mediate SDIA of mouse ES cells into neural cell lineage. The neural progenitor cell line together with two stromal cell lines, L and L-Wnt-3A, were molecularly analyzed for neurotrophic and neuro-protective factors. Derived cell products were immunologically and molecularly characterized for neural lineage commitment. *In-vivo* studies of SDIA-induced products were conducted in the mouse model of brain ischaemia induced by transient BCCAO and reperfusion. Behavioural assessment of ischaemic mice post-transplant into the striatum was performed in the water maze system. Tracking of bromodeoxyuridine-labelled products was made on brain sections and teratoma was examined.

Figure 1 and Figure 2 depict the strategic plan and analyses of the *in-vitro* and *in-vivo* studies in the project. The maintenance culture condition of undifferentiated mouse ES cell lines, D3 and E14TG2a, were optimized by testing the effects of three different serum from two companies (Gibco and Hyclone), and two different feeders PMEF and STO on the cell number, cell viability and SSEA-1 positivity.

Subsequently the undifferentiated ES cells were propagated for differentiation-induction of the neural lineage. Two main strategies were used. The first one involved the formation of embryoid bodies from ES cells under the influence of retinoic acid. The

second one involved the use of SDIA, in which ES cells were co-cultured with three different cell lines, a neural progenitor cell line C17.2 and an adiposal cell line and its derivative, L cells and L-Wnt-3A cells. The ES-derived products were characterized morphologically, immunologically and genetically.

After *in-vitro* induction, the functionality of the SDIA-induced cell products, which has the largest proportion of nestin-positive colonies, was investigated in the ischaemic-induced mice model. BrdU-labelled cell products were intracranially implanted onto the straitum of six ischaemic mice. Behavioral improvements of three groups of mice, including six normal mice, six ischaemic mice and six transplanted ischaemic mice, were assessed by a water maze system for 5 days. After assessment, all mice were sacrified and their brains were isolated and stored in either OCT medium or paraffin blocks. Sections at different positions were made and were subjected to hematoxylin and eosin staining and BrdU-immunostaining for histological analysis.

Figure 1 The scheme of *in-vitro* studies

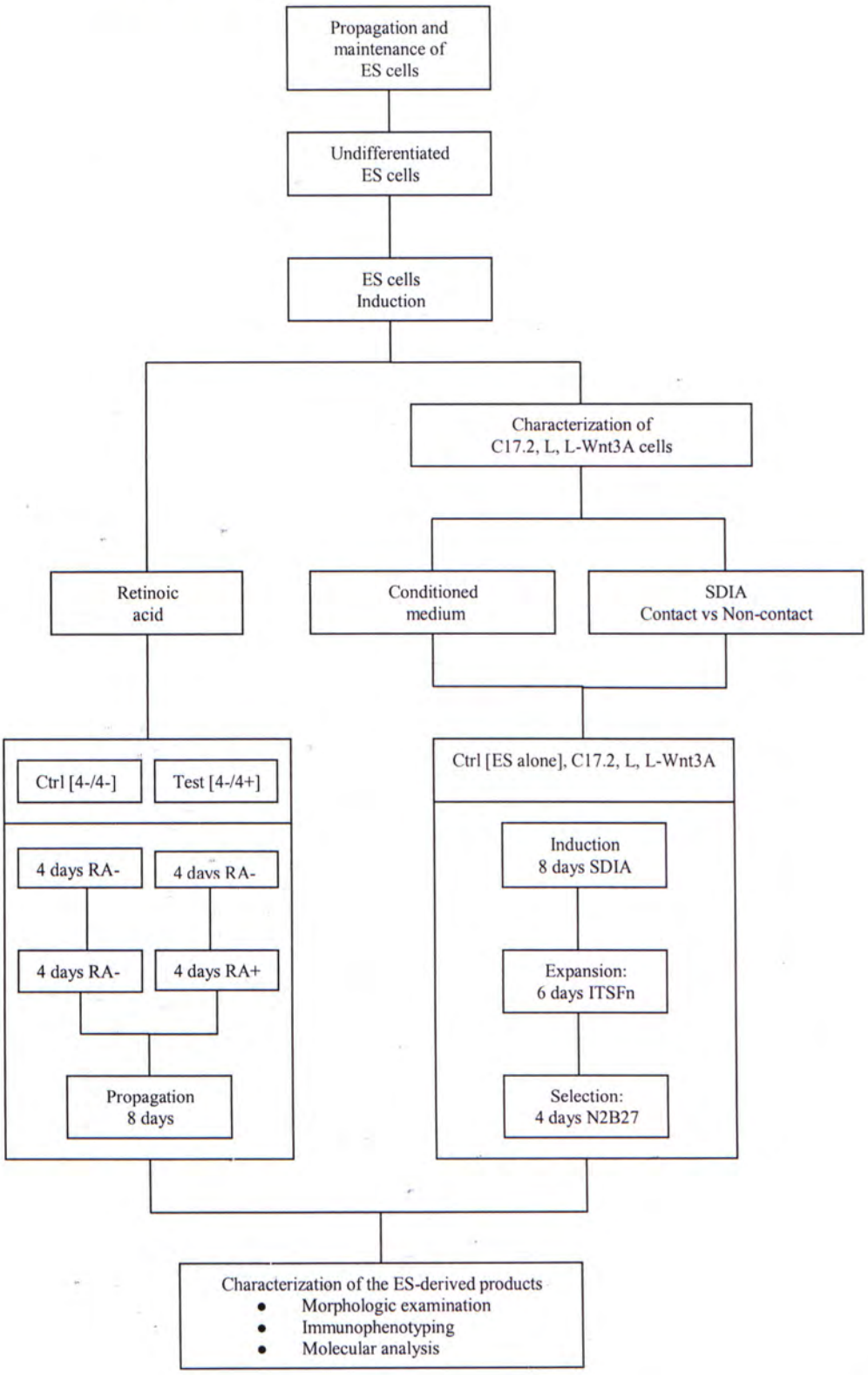
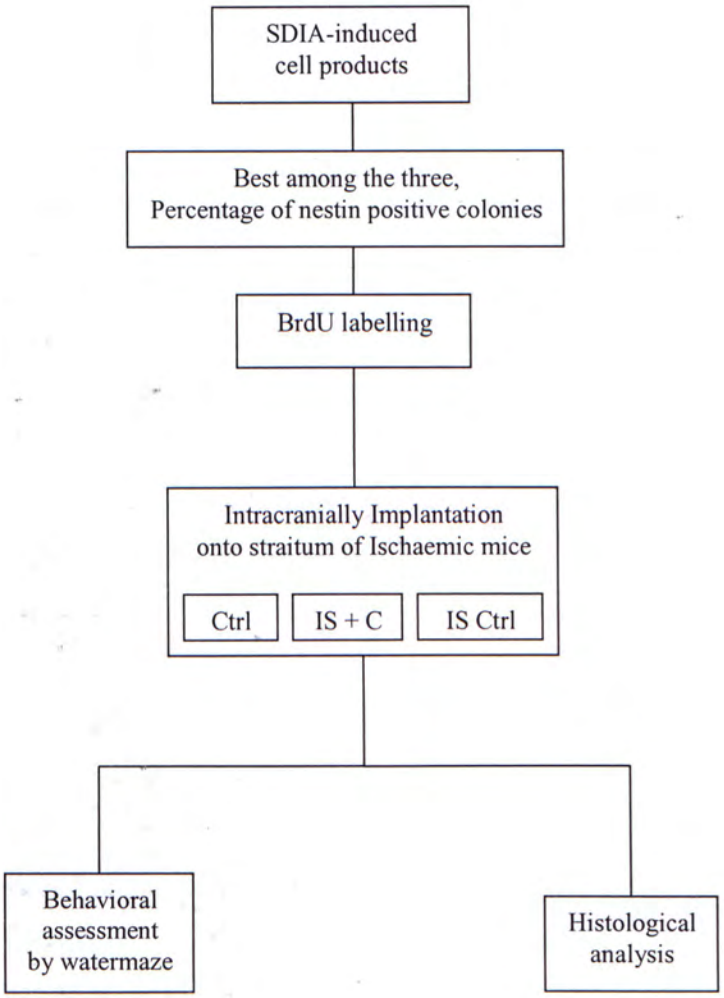


Figure 2 The scheme of *in-vivo* studies



Chapter 3 Materials and Methods

3.1 Chemicals and Reagents

3.1.1 Cell Culture

The followings were used for propagation, induction and differentiation of ES cells.

- Acetic acid, glacial (Cat. 1.00063.2500, Merck & Co., Inc, USA)
- All-*trans* Retinoic acid, powder (Cat. R2625, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- B27 supplement, 50X (Cat. 17504-044, Gibco, Invitrogen Corporation, London, UK)
- Dimethyl sulfoxide (DMSO; Cat. WAK-DMSO-10, Cryosure, Amphi Ltd., Turkey)
- Dulbecco's Phosphate-Buffered Saline, 1X, liquid (Cat. 14190-136, Gibco, Invitrogen Corporation, London, UK)
- Fibronectin from bovine plasma, 0.1% (Cat. F1141, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Foetal calf serum, Qualified, 500mL (Cat. 26140-079, Gibco, Invitrogen Corporation, London, UK)
- Gelatin powder (Cat. 101186M, BDH, Merck & Co., Inc, USA)
- Geneticin, Selective Antibiotic, powder (Cat. 11811-031, Gibco, Invitrogen Corporation, London, UK)
- Glycerol (Cat. 101186M, BDH, Merck & Co., Inc, USA)
- Hamilton syringe, 10uL (Cat. 80330, Hamilton Company, Nevada, USA)
- Insulin-Transferrin-sodium Selenite media supplement, powder (Cat. I1884, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Ketamine, 10% (Alfasan, FarmaVet SA, Romania)

- Knockout Dulbecco's Modified Eagle's Medium (Cat. 10829-018, Gibco, Invitrogen Corporation, London, UK)
- Knockout Serum Replacer (Cat. 10828-028, Gibco, Invitrogen Corporation, London, UK)
- Laminin from Engelbreth-Holm-Swarm Murine sarcoma (basement membrane) (Cat. L2020, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Leukemia inhibitory factors (Cat. CMI-LIF2010, Chemicon, Chemicon International, Inc., Boronia Victoria, Australia)
- L-Glutamine-200 mM (100X), liquid (Cat. 14190-136, Gibco, Invitrogen Corporation, London, UK)
- MEM Non-Essential Amino Acids Solution 10 mM (100X), liquid (Cat. 11140-050, Gibco, Invitrogen Corporation, London, UK)
- Mercaptoethanol, beta (Cat. M7522, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Mitomycin C from Streptomyces caespitosus, powder (Cat. M4287, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- N2 supplement, 100X (Cat. 17502-048, Gibco, Invitrogen Corporation, London, UK)
- Neurobasal medium (Cat. 21103-049, Gibco, Invitrogen Corporation, London, UK)
- Penicillin-Streptomycin, liquid (Cat. 15140-122, Gibco, Invitrogen Corporation, London, UK)
- Poly-L-ornithine Solution 0.01%, Sterile-filtered (Cat. P4957, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Trypan Blue (Cat. 07050, Stem Cell Technologies, North America)
- Trypsin-Ethylenediaminetetraacetic Acid (EDTA), 0.05%, 1X (Cat. 25300-054, Gibco, Invitrogen Corporation, London, UK)
- Trypsin-EDTA, 0.25%, 1X (Cat. 15050-057, Gibco, Invitrogen Corporation, London, UK)
- Xylazine, 2% (Alfasan, FarmaVet SA, Romania)

3.1.2 Immunohistochemistry and staining

The followings were for immunophenotyping and staining of ES cells and their derived products.

- 4', 6'-diamidino-2-phenylindole hydrochloride (Cat. H1200, Vector Laboratory Inc., California, USA)
- Aminopropyl triethoxy-silane, APES (Cat. A3648, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- BrdU, colorimetric, 1000X (Cat. 1647229, Roche Applied Science, Basel, Switzerland)
- Eosin Yellowish (Cat. 34197, BDH, Merck & Co., Inc, USA)
- Fluorescein isothiocyanate (FITC) anti-BrdU (Cat. 1202693, Roche Applied Science, Basel, Switzerland)
- FITC Goat anti mouse IgG (H+L) Conjugate (Cat. 816511, Zymed Laboratory Inc. (Invitrogen Corporation), London, UK)
- FITC Goat anti mouse IgM (m chain specific; Cat. 626811, Zymed Laboratory Inc. (Invitrogen Corporation), London, UK)
- Normal Goat Serum (Cat. 01-6201, Zymed Laboratory Inc. (Invitrogen Corporation), London, UK)
- Haematoxylin Hydrate (Cat. 34037, BDH, Merck & Co., Inc, USA)
- Monoclonal antibody to TuJ III perufied antibody (Cat. 11-264-C100, Exbio antibodies, Vestec Czech Republic)
- Monoclonal mouse anti-GFAP antibody (Cat. 18-0021, Zymed Laboratory Inc. (Invitrogen Corporation), London, UK)
- Monoclonal mouse anti-MAP-2 antibody (Cat. 131500, Zymed Laboratory Inc. (Invitrogen Corporation), London, UK)
- Monoclonal mouse anti-Nestin antibody (Cat. 611659, Pharmingen, BD Transduction Laboratories, CA, USA)
- Monoclonal Mouse anti-Oct-4 antibody (Cat. sc-5279, Santa Cruz, Santa Cruz Biotechnology Inc., CA, USA)

- Monoclonal Mouse anti-SSEA-1 antibody (Cat. sc-21702, Santa Cruz, Santa Cruz Biotechnology Inc., CA, USA)
- Monoclonal Mouse anti-Tumor rejecting antigen 1-81 (Tra1-81) antibody (Cat. sc-21706, Santa Cruz, Santa Cruz Biotechnology Inc., CA, USA)
- Mouse anti-TH antibody (Cat. 32-2100, Zymed Laboratory Inc. (Invitrogen Corporation), London, UK)
- Paraformaldehyde, crystalline (Cat. P6148, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Phenylenediamine (Cat. P6001, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Popidium Iodide (Cat. P3556, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Purified anti-human MBP antibody (Cat. 559904, Pharmingen, BD Bioscience, CA, USA)
- Tetramethylrhodamine isothiocyanate (TRITC) goat anti-mouse IgG (H+L) Conjugate (Cat. 816514, Zymed Laboratory Inc. (Invitrogen Corporation), London, UK)
- Triton X-100 (Cat. T8787, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Xylene (Cat. 8681, Merck & Co., Inc, USA)

3.1.3 Molecular Biology

The followings were used for RNA extraction, reverse transcriptase polymerase chain reaction (RT PCR) and visualization of gene products.

- 10X PCR buffer-$MgCl_2$ (Cat. Y02028, Gibco, Invitrogen Corporation, UK)
- 10X Stratascript (Cat. 600085-52, Stratagene Corporation, California, USA)
- DNA ladder (Cat. SM0243, MBI, Fermentas Life Sciences, Burlington, Canada)
- Deoxynucleoside Triphosphate (Cat. R0192, MBI, Fermentas Life Sciences, Burlington, Canada)

- Eithidium bromide (Cat. 2515, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- $MgCl_2$ 50mM (Cat. Y02016, Gibco, Invitrogen Corporation, London, UK)
- Platinum Taq DNA polymerase (Cat. 10966-034, Gibco BRL, Invitrogen Corporation, London, UK)
- Reverse Transcriptase (Cat. 600085-51, Stratagene Corporation, California, USA)
- Rnase inhibitor (Cat. N211B, Promega Corporation, Madison (New York), USA)
- Ribonucleic acid (RNA) RNeasy Extraction Mini Kit (Cat. 74106, Qiagen Pty Ltd, Victoria, Australia)
- Superscript II Rnase H (Cat. 18064-071, Gibco, Invitrogen Corporation, London, UK)
- Tris-Acetic acid-EDTA Buffer Solution, TAE, 50X (Cat. 24710-030, Gibco, Invitrogen Corporation, London, UK)
- Tween-20 (Cat. P1379, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)

3.2 Consumable

The following items were general consumables used in this project.

- Flasks Nunclon with Filter Caps, 80cm^2, Polystyrene, Sterile (Cat. 178891, Nunc, Denmark)
- Tissue culture dish,100 x 20mm (Cat. 430167, Corning Incorporated, New York, USA)
- Culture plate, 6-well, with lids, sterile (Cat. 140675, Multidishes Nunclon, Nunc, Denmark)
- Culture plate, 12-well, with lids, sterile (Cat. 140675, Multidishes Nunclon, Nunc, Denmark)
- Culture plate, 24-well, with lids, sterile (Cat. 142475, Multidishes Nunclon, Nunc, Denmark)

- Ultra-low attachment polystyrene 6-well culture plate (Cat. 3471, Corning Incorporated, New York, USA)
- Ultra-low attachment polystyrene 24-well culture plate (Cat. 3473, Corning Incorporated, New York, USA)
- Petri dish, with triple vents, 94 x 15mm (Cat. 633185, Greiner, Greiner Bio-one, Frickenhausen, Germany)
- Non-treated Flasks, 25cm^2, Polystyrene, Sterile (Cat. 169900, Nunc, Denmark)
- Thincert cell culture insert for 12 well plates with transparent membrane (PET) ,with pore size: 0.4 μm (Cat. 16670, Greiner, Greiner Bio-one, Frickenhausen, Germany)
- Thincert cell culture insert for 6 well plates with transparent membrane (PET) ,with pore size: 0.4 μm (Cat. 16669, Greiner, Greiner Bio-one, Frickenhausen, Germany)
- Cell culture flask, 75cm^2, with filter, sterile (Cat. 658175, Greiner, Greiner Bio-one, Frickenhausen, Germany)
- Cell culture flask, 25cm^2,with filter, sterile (Cat. 690275, Greiner, Greiner Bio-one, Frickenhausen, Germany)
- Pipette, 2mL (Cat. PN2E1, Orange, AM Plus Company, HK)
- Pipette, 5mL (Cat. PN5E1, Orange, AM Plus Company, HK)
- Pipette, 10mL (Cat. PN10E1, Orange, AM Plus Company, HK)
- Polypropylene Round-Bottom Test Tube, snap cap, 5 mL, 12x75 mm (Cat. 352063, BD Falcon, BD Biosciences, CA, USA)
- Universal bottle (Cat. BS128A, Sterilin, Barloworld Scientific Ltd., Staffordshire, UK)
- 50mL Centrifuge tube, Polypropylene (Cat. 210261, Greiner, Greiner Bio-one, Frickenhausen, Germany)
- Cell Strainer, 40um (Cat. 352340, BD Falcon, BD Biosciences, CA, USA)
- Cell Scrapers (Cat. 179707, Nunc, Denmark)
- Cryotube, internal thread, Polypropylene, and screw stopper, Sterile (Cat. 377267, Nunc, Denmark)
- Cover glass, 12mm circular (Cat. CM120RA1, Gerhard Menzel ,Germany)
- Parafilm (Cat. PM996, Parafilm, South East Chem. and Instru Ltd, HK)

- Syringe, 25 gauge, 1mL (Cat. BND305125, Becton Dickinson, BD Bioscience, New Jersey, USA)
- Filter Syringe 0.22μm Polyethersulfone, Sterile (Cat. SLGP033RS, Millipore Corporation, Billerica, USA)
- Microscope slides (Cat. 7101, Sail Brand, China)

3.3 Cell lines

3.3.1 Feeder cells

3.3.1.1 Primary mouse embryonic fibroblasts

Primary mouse embryonic fibroblasts (PMEF) were isolated from embryos of C57BL/6 pregnant mice 13.5 to 14.5 days post coitus (dpc). They were eight to nine weeks old and were bred in the Laboratory Animal Services Centre of the Chinese University of Hong Kong. The appearance of vaginal plugs was designated as day 0 of gestation. PMEF were maintained at a relatively high density of 1 x 10^6/mL. PMEF which were passaged for three to six times were ready for use.

3.3.1.2 STO

STO (CRL-1503, American Type Culture Collection, Manassas, Virginia, USA) was derived from a continuous line of SIM mouse embryonic fibroblasts. They are adhesive and fibroblast in morphology.

3.3.1.3 L Cells

L Cells (CRL-2648, American Type Culture Collection, Manassas, Virginia, USA) is a cell line isolated from the subcutaneous connective tissue, areolar and adipose of C3H/An strain mice. They are adhesive and fibroblast in morphology. It is the parental line for the L Wnt-3A cell line, which was transfected with Wnt-3A expression vector and acts as a control of L-Wnt-3A.

3.3.1.4 L-Wnt-3A Cells

L-Wnt-3A (CRL-2648, American Type Culture Collection, Manassas, Virginia, USA) secretes the biologically active Wnt-3A protein and was established from a stable clone of L cells transfected with Wnt-3A expression vector in G418 selective medium. The Wnt-3A gene encodes a secreted glycoprotein with a variety of signaling effects. Wnt genes control many patterning and growth events during embryonic development. They are adhesive and fibroblast in morphology.

3.3.1.5 C17.2

C17.2 is a cell line that generated by retrovirus-mediated v-myc transfer into progenitors cultured from neonatal mouse cerebellum. They are adhesive and neuronal morphology. The multipotent neural progenitor cell line was a kind gift from the late Dr. David Walsh, Department of Anatomy, University of New South Wales, Australia.

3.3.2 ES cells

3.3.2.1 ES-D3

ES-D3 (CRL-1934, American Type Culture Collection, Manassas, Virginia, USA) is a mouse ES cell line isolated from the blastocyst of 129/Sv+c/+p strain mice. Single undifferentiated D3 is epithelial in morphology. When injected into blastocysts, D3 can colonize the germline and reconstitute a mouse. The number of passage was not available.

3.3.2.2 ES-E14TG2a

ES-E14TG2a (CRL-1821, American Type Culture Collection, Manassas, Virginia, USA) is a mouse ES cell line isolated from the blastocyst of 129/Ola strain mice. Single undifferentiated E14TG2a is spherical in morphology. The cells are deficient in hypoxanthine guanine phosphoribosyl transferase and are resistant to 0.06 mM 6-thioguanine. When injected into blastocysts, E14TG2a can colonize the germline. The number of passage was not available.

3.4 In-house prepared solutions

3.4.1 Stock solution of Insulin, Transferrin, Selentine (ITS) Supplement

ITS supplement powder (Cat. I1884, Sigma)	5g
Glacial acetic acid (Cat. 1.00063.2500, Merck)	0.2mL
Sterile double-deionized water	5mL

The solution was filter-sterilized and made up to volume of 50 mL with sterile double-deionized water. Aliquots were kept at -80°C until use for no more than 3 months.

3.4.2 Enriched Knock-Out Dulbecco's Modified Eagle's Medium (KO DMEM)

KO DMEM (Cat. 10829-018, Gibco)	500mL
L-Glutamine-200 mM, 100X (Cat. 14190-136, Gibco)	5mL
Penicillin-Streptomycin (Cat. 15140-122, Gibco)	5mL

Aliquots were kept at -80°C until use for no more than 1 month.

3.4.3 Mitomycin C solution

Mitomycin C (Sigma, USA)	2mg
Enriched KO DMEM	10mL

The solution was filter-sterilized. Aliquots were kept at -80°C until use for no more than 30 days.

3.4.4 Gelatin solution 0.1%

Gelatin powder (Merck & Co., USA)	0.5g
Sterile double-deionized water	500 mL

The solution was warmed at 37°C until complete dissolution. The solution was autoclaved and aliquoted were kept at -80°C until use for no more than 30 days.

3.4.5 β-mercaptoethanol solution

3.4.5.1 β-mercaptoethanol solution 0.1M

β-mercaptoethanol (14.33M) (Cat. M7522, Sigma)	1mL
Sterile double-deionized water	13.3mL

3.4.5.2 β-mercaptoethanol solution 0.1M

1M β-mercaptoethanol	1mL
Sterile double-deionized water	9mL

3.4.5.3 β-mercaptoethanol solution 0.1M for preparation of culture medium

0.1M β-mercaptoethanol	1mL
Enriched KO DMEM	9mL

The solution was freshly prepared for preparation of medium.

3.4.6 ALL-*trans* retinoic acid

3.4.6.1 ALL-*trans* retinoic acid stock solution 0.01M

ALL-*trans* RA powder (Molecular Weight = 300.4; Cat. R2625, Sigma)	0.03g
Absolute ethanol	10mL

The solution was filter-sterilized. Aliquots were kept at -80°C until use for no more than 14 days.

3.4.6.2 ALL-*trans* retinoic acid working solution 1μM

0.01M ALL-*trans* RA stock	100ul
Dulbecco's Phosphate-Buffered Saline (DPBS; Cat. 14190-136, Gibco)	9.9mL

Aliquots were kept at -80°C in dark until use for no more than 7 days.

3.4.7 Paraformaldehyde solution 4% (PFA)

Paraformaldehyde (PFA; Cat. P6148, Sigma)	4g
DPBS (Cat. 14190-136, Gibco)	100mL

The solution was warmed at 55°C-60°C until complete dissolution. The solution was kept in dark at -80°C until use for no more than 30 days.

3.4.8 Tritox X-100 solution

3.4.8.1 Tritox X-100 solution 3%

Triton X-100, 100% (Cat. T8787, Sigma)	300ul
DPBS (Cat. 14190-136, Gibco)	9.7mL

The solution was kept in dark at room temperature until use for no more than 30 days.

3.4.8.2 Tritox X-100 solution 0.3%

3% Triton X-100	1mL
DPBS (Cat. 14190-136, Gibco)	9mL

The solution was kept in dark at room temperature until use for no more than 30 days.

3.4.9 Popidium iodide solution 1ug/mL (PI)

Phenylenediamine (PI; Cat. P6001, Sigma)	100mg
Glycerol (Cat. 101186M, BDH)	90mL
DPBS (Cat. 14190-136, Gibco)	10mL
Popidium Iodide (Cat. P3556, Sigma)	10ul

The solution was stirred thoroughly and pH was adjusted to 8.0 with 0.5M hydrocarbonate acid pH9.0. Aliquots were kept at -20°C in dark until use for no more than 1 year

3.4.10 Geneticin solution

3.4.10.1 Geneticin solution 50mg/mL

G418 powder (Cat. 11811-031, Gibco)	50mg
DPBS (Cat. 14190-136, Gibco)	10mL

The solution was filter-sterilized. Aliquots were kept at -80°C until use for no more than 14 days.

3.4.10.2 Geneticin solution 5mg/mL

Geneticin solution 50mg/mL	1mL
DPBS (Cat. 14190-136, Gibco)	9mL

Aliquots were kept at -80°C until use for no more than 14 days.

3.4.11 Poly-L-ornithine solution

Poly-L-ornithine Solution 0.01% (Cat. P4957, Sigma)	4mL
Sterile double-deionized water	22.6mL

3.4.12 Laminin solution

Laminin (Cat. L2020, Sigma)	25uL
DPBS (Cat. 14190-136, Gibco)	25mL

3.4.13 Maintenance medium for cell feeders

KO DMEM (Cat. 10829-018, Gibco)	400mL
Foetal Calf Serum (FCS), qualified (Cat. 26140-079, Gibco)	75mL
L-Glutamine-200 mM, 100X (Cat. 14190-136, Gibco)	5mL
Penicillin-Streptomycin, (Cat. 15140-122, Gibco)	5mL
Non-Essential AA Solution 10 mM, 100X (Cat. 11140-050, Gibco)	5mL
β-mercaptoethanol (0.1M)	10mL

Aliquots were kept at -20°C until use for no more than 1 month.

3.4.14 Mitomycin C inactivation medium

KO DMEM (Cat. 10829-018, Gibco)	450mL
FCS, qualified (Cat. 26140-079, Gibco)	25mL
L-Glutamine-200 mM, 100X (Cat. 14190-136, Gibco)	5mL
Penicillin-Streptomycin, (Cat. 15140-122, Gibco)	5mL
Non-Essential AA Solution 10 mM, 100X (Cat. 11140-050, Gibco)	5mL
β-mercaptoethanol (0.1M)	10mL

Aliquots were kept at -20°C until use for no more than 1 month.

3.4.15 Freezing medium

KO DMEM (Cat. 10829-018, Gibco)	4mL
FCS, qualified (Cat. 26140-079, Gibco)	4mL
DMSO (Cat. WAK-DMSO-10, Cryosure)	2mL

The freezing medium was freshly prepared. DMSO was added to the chilled medium.

3.4.16 Propagation medium for ES cells

There are two available propagation media for ES cells namely; serum-based medium allowing a rapid growth but more difficult to control the undifferentiated status and serum-free medium yielding in a slower growth.

3.4.16.1 Serum-based propagation medium for ES cells

KO DMEM (Cat. 10829-018, Gibco)	400mL
ES Cell-Qualified FCS (Cat. 10439-024, Gibco)	75mL
L-Glutamine-200 mM, 100X (Cat. 14190-136, Gibco)	5mL
Penicillin-Streptomycin, (Cat. 15140-122, Gibco)	5mL
Non-Essential AA Solution 10 mM (100X)(Cat. 11140-050, Gibco)	5mL
β-mercaptoethanol (0.1M)	10mL

Aliquots were kept at -20°C until use for no more than 1 month. 1000IU/mL LIF (Cat. CMI-LIf2010, Chemicon) was added before used.

3.4.16.2 Serum-free propagation medium for ES cells

KO DMEM (Cat. 10829-018, Gibco)	400mL
Knock Out Serum Replacor (SR; Cat. 10828-028, Gibco)	75mL
L-Glutamine-200 mM, 100X (Cat. 14190-136, Gibco)	5mL
Penicillin-Streptomycin, (Cat. 15140-122, Gibco)	5mL
Non-Essential AA Solution 10 mM, 100X (Cat. 11140-050, Gibco)	5mL
Beta mercaptoethanol (0.1M)	10mL

Aliquots were kept at -20°C until use for no more than 1 month. 1000IU/mL LIF was added before used.

3.4.16.3 Serum-free induction medium for ES cells

3.4.16.3.1 Serum-free induction medium I

KO DMEM (Cat. 10829-018, Gibco)	410mL
KO SR (Cat. 10828-028, Gibco)	75mL
L-Glutamine-200 mM, 100X (Cat. 14190-136, Gibco)	5mL
Penicillin-Streptomycin, (Cat. 15140-122, Gibco)	5mL
Non-Essential AA Solution 10 mM, 100X (Cat. 11140-050, Gibco)	5mL

Aliquots were kept at -20°C until use for no more than 1 month.

3.4.16.3.2 Serum-free induction medium II

Insulin, Transferrin, Selentine and Fibronectin (ITSFn) medium	
Enriched KO DMEM	98.9mL
Insulin, Transferrin, Selentine (ITS) Supplement	1mL
Fibronectin (Fn) from bovine plasma (Cat. F1141, Sigma)	100uL

The ITSFn medium was freshly prepared by supplementing fibronectin solution immediately before use.

3.4.16.3.3 Serum-free induction medium III

N2-/B27-supplemented Neurobasal medium (N2B27)	
Neurobasal medium (Cat. 21103-049, Gibco)	38.8mL
L-Glutamine-200 mM, 100X (Cat. 14190-136, Gibco)	0.4mL
Penicillin-Streptomycin, (Cat. 15140-122, Gibco)	0.4mL
B27 supplement, 50X (Cat. 17504-044, Gibco)	80uL
N2 supplement, 100X (Cat. 17502-048, Gibco)	320uL

The N2B27 medium should be kept at 4°C, and used within 1 week.

3.5 Equipments

Below were equipments used throughout the whole project.

- Bench-top Centrifuge (Model: CR412, Jouan, Thermo Electron's Corporation, New York, USA)
- Ohaus Analytical Plus Balance (Model: AP250D, OHAUS, New Jersey, USA)
- Ohaus Harvard Trip Balance (Model:1450-SD, OHAUS, New Jersey, USA)
- Water Jacketed CO2 Incubator with HEPA filter (Model: Series II 3111, Thermo Forma, Thermo Electron's Corporation, New York, USA)
- Biological safety cabinet (Model: 1185, Thermo Forma, Forma Scientific, Inc., New York, USA)
- Inverted microscope (Model: IX71, Olympus, Olympus Corporation, Tokyo, Japan)
- Coulter haematology analyzer (Model: AcT Diff, Coulter Corporation, Miami, USA).
- Spectrophotometer (GeneQuant II, Pharmacia LKB Biochrom Ltd., Cambridge, England)
- Programmable Thermal controller (Model: PTC-100, Hoefer Scientific Instruments, San Franciso, USA)
- Vortex Mixer (Model: M37615, Thermolyne, Barnstead International, USA)
- Bench top and refrigerated centrifuges (Model: 5417c, Eppendorf International, Hamburg, Germany)
- Rotary Microtome (Model: RM2035, Leica, Germany)
- Watermaze System (Model: 1030120 (SH-1), Shanghai Xi Bo Biotechnology Ltd, Shanghai, China)
- Stereotaxic Frame (Model: 900, KOPF, California, USA)
- Mouse Adaptor (Model: 921-E, KOPF, California, USA)
- Mouse Ear Bars (Model: 921-F&G, KOPF, California, USA)
- Volvere-GX, handpieces (Model: GX-35EM, Nakanishi, Inc, Japan)
- Laboratory gasburner, gasprofi 1SCS (Model.6.103.000, WLD-TEC, Techcomp Ltd., North America)
- Cytocentrifuge (Model: Autosmear CF-120, Sakura Seiki Co., Tokyo, Japan)

- γ-irradiator (Gammacell 1000 Elite #214, MDS Nordion, Canada)
- Neubauer hemocytometer, improved
- Instant camera (Polaroid DS34, St. Albans, Hertfordshire, England)
- Electrophoresis documentation and analysis system (Eastman Kodak Company, Rochester, NY, USA)

3.6 Methods

3.6.1 Cell Culture

All culture works were aseptically performed in a biological safety cabinet. Stringent measures were implemented to avoid microbial contamination and cross infection according to the policies and procedures stipulated by the Chinese University of Hong Kong and Prince of Wales Hospital, Hospital Authority.

3.6.1.1 Preparation of round cover-slips

Round glass covers-lips of 12-mm diameter were dipped into 70% ethanol and then flamed in Bunsen burner. Sterilized cover-slips were individually and aseptically transferred into wells of 24-well tissue culture plate.

3.6.1.2 Gelatinization of tissue culture wares

Coating of tissue culture wares with gelatin facilitates better adhesion. Three mL of 0.1% gelatin solution were distilled into 25cm^2 flasks, whereas six mL were dispensed to 75cm^2 flasks or 100 mm tissue culture dishes. They were allowed to stand for at least 20 minutes at ambient temperature. Gelatin solution was aspirated off. Air-dried tissue wares were kept at 4°C until use for no more than a month (Pollard 1997; Zigova et al. 2002).

3.6.1.3 Poly-L-ornithine and laminin coating

One mL of freshly prepared 0.01% poly-L-ornithine in sterile doubled de-ionized water was dispensed into each well of tissue culture plates with or without the insertion of sterile round cover-slip. The plate was incubated for an hour at 37°C and was then washed thrice with sterile DPBS for five minutes each. One mL of 1ug/mL laminin in sterile DPBS was added. The plate was further incubated for an hour at 37°C. Upon completion of washing with DPBS, plate was ready for use.

3.6.1.4 Thawing frozen cells

A frozen ampoule of stromal feeder cells wrapped in a sterile plastic bag was quickly thawed within 2 minutes in a 37°C-water bath with gentle agitation. The de-froze cell suspension were diluted drop-wise with 10mL of ice-cold fibroblast medium

and allowed to equilibrate for 10 minutes at 4°C. Thereafter, cell suspension was spun at 250 x g for eight minutes at ambient temperature and supernatant was discarded. The cell pellet was re-suspended and adjusted to 1 x 10^5/mL with fibroblast medium and plated onto gelatinized 75 cm^2 flasks or 100mm tissue culture dishes. Cultures were maintained at 37°C in a humidified 5% CO_2-incubator. Medium was changed overnight and cultures were maintained until confluency.

3.6.1.5 Passage of adherent culture

Confluent cultures of adherent cells were washed twice with DPBS and were enzymatically digested with 0.05% trypsin containing 0.53 μM EDTA at 37°C for five minutes or until cell detachment was evident. Trypsinization was stopped with twice volumes of fibroblast medium. Cell suspension was dislodged and triturated using a fine-pored pipette. Cells were counted.

3.6.1.6 Cell count

Automated cell counts were generated using the Coulter haematology analyzer. Cell suspensions of counts below the sensitivity limit were counted manually using an improved Neubauer hemocytometer under a microscope. Appropriate dilution of cell suspension was made so that each mm^2 grid area of the four corners accommodated approximately 100 cells. Cells in the eight mm^2 area were counted using 10x objective of microscope. The mean cell concentration was calculated.

3.6.1.7 Cytospin

Cell suspension was adjusted to 1 x 10^6/mL in DPBS supplemented with 1% bovine serum albumin. A hundred uL of the cell suspension was dispensed to the cyto-housing and spun at 25 x g for five minutes onto a glass slide. Slides were air-dried, wrapped with aluminum foil and kept at -80°C until analysis.

3.6.1.8 Cell viability test

One fifth volume of tryphan blue dye solution was added to the cell suspension. A minimum of 500 unstained viable cells and stained dead cells were counted using an improved Neubauer hemocytometer. Cell viability was expressed as a percentage of the total cells counted.

3.6.1.9 Cryopreservation

Cell suspension of 2 x 10^6/mL was mixed with equal volume of freezing medium and was aliquoted into cryo-vials. They were then put into a pre-cooled styrofoam box and kept in an ultra-low freezer at -80°C freezer overnight. Frozen cells were transferred to a liquid nitrogen tank for long-term storage.

3.6.1.10 Preparation of primary mouse embryonic fibroblast (PMEF)

Mouse embryonic fibroblasts were isolated from embryos derived from pregnant mice 13.5 to 14.5 dpc supplied by the Laboratory Animal Services Centre of the Chinese University of Hong Kong. Mice were sacrificed by cervical dislocation. The mice were laid on its back and the abdomen was disinfected with 70% ethanol. With sterilized dissecting instruments (fine scissors, tweezers, razor blades and forceps), the uterine horns were dissected. They were rinsed briefly in 70% ethanol and placed onto a Petri dish containing sterile DPBS. Embryos were separated from the placenta and surrounding membranes and transferred to a second Petri dish with sterile DPBS. The head, liver and heart of the embryo were carefully removed. The remaining body was washed with fresh DPBS to remove as much blood as possible. In a minimal volume of DPBS, the embryonic body was finely minced into many small pieces using razor blades and scissors until being pipettable. Excessive DPBS was removed after centrifugation at 250 x g for 10 minutes. The cell suspension was enzymatically digested in 0.25% trypsin solution containing 0.53 μM EDTA for approximately 15 minutes in a 37°C water bath with constant agitation. At five-minute intervals, the cell suspension was vortexed for 30 seconds. Trypsinization was stopped by adding double volumes of fibroblast medium. Tissues in large pieces were mechanically dissociated by pipetting up and down through a 5-mL pipette. The cell suspension was allowed to stand for two minutes to sediment large pieces of cellular aggregates. The supernatant was transferred into a new tube and pelleted at 250 x g for 10 minutes. Cells were re-suspended in fresh medium and triturated through a fine-pored pipette. Cells segregated from five embryos

were sufficient to seed a $75cm^2$ flask and cultures were incubated for 2-3 days at 37°C in a humidified 5% CO_2-incubator. Confluent growth of PMEF, which was regarded as P0, took place in three to four days. Cultures were then passaged, split into two to three flasks or kept as frozen stock (Joyner 1999).

3.6.1.11 Mitomycin C inactivation of feeder cells

Cultures of approximately 90% confluency were treated with 10 μg/mL Mitomycin C for 2.5 hours to arrest cell division by inhibiting DNA synthesis and nuclear division. Treated cultures were washed twice with DPBS and trypsinized. Cell suspensions of 8×10^5, 1.5×10^6 and 3×10^6 in fibroblast medium were seeded onto 12-well, 6-well culture insert and gelatinized $75cm^2$ flask or 100-mm tissue culture dish, respectively, and cultured for at least three hours to overnight at 37°C in a humidified 5% CO_2-incubator (Joyner 1999). Upon establishment of the feeder layer onto the culture ware, cultures were washed once with DPBS before seeding ES cells or proceeding to the preparation of CM.

3.6.1.12 Gamma irradiation of various feeders

Trypsinized cell suspensions in a 50-mL tube were exposed to 60-Gray irradiation in the γ-irradiator. Irradiated cell products were seeded and cultured under the same condition of mitomycin C-inactivated cell suspensions.

3.6.1.13 Preparation of CM from feeder cells

Having washed with DPBS, cultures of mitomycin C-inactivated or irradiated feeder cells were replenished with serum-free propagation medium and maintained for four days at 37°C in a humidified 5% CO_2-incubator. The CM was collected and spun at 400 x g for 10 minutes to pellet cellular debris. The supernatant was frozen at -80°C. Collections from six batches of CM were pooled and used thereafter.

3.6.1.14 Propagation of ES cells in serum-based medium

ES cell lines, D3 and E14TG2a, were cultured according to the package inserts. Frozen ES cells were thawed in 15 mL of the serum-based propagation medium containing LIF at 1000 units/mL. They were directly plated onto gelatinized culture ware pre-established with the inactivated feeder cells of PMEF or STO, and were incubated at 37°C in a humidified 5% CO_2-incubator. Medium was changed once or twice depending on the growth of ES cells and culture was passaged every two days (Joyner 1999).

3.6.1.15 Propagation of ES cell in serum-free medium

ES cells were thawed and cultured as above-mentioned. On the day of passage, ES cells were trypsinized and were replated in 10 mL serum-free propagation medium supplemented with LIF at 1000 units/mL onto 75cm^2 flask or 100-mm tissue culture dish

for 45 minutes to an hour to deplete PMEF. Non-adherent cells were enriched upon centrifugation at 250 x g for 10 minutes. Cell pellet was dislodged and resuspended. Viable ES cells of refractile discoid mononuclear morphology and scanty cytoplasm were microscopically counted in an improved Neubauer hemocytometer. Re-plating was carried out if the proportion of ES cells was below 60%. Enriched ES cells at 1 x 10^5/mL serum-free propagation serum with 1000 units/mL LIF were seeded onto gelatinized culture ware pre-established with the inactivated feeder cells.

ES cell suspensions of 1 x 10^5/ml in 3 mL, 5 mL and 10 mL were seeded onto well of ultra-low adherent 6-well plate, low binding 25cm^2 flask and 100-mm Petri dish, respectively, without feeder cells. They were incubated at 37°C in a humidified 5% CO_2-incubator. Medium was changed once or twice by discarding spent medium after centrifugation at 250 x g for 10 minutes and cell pellet was re-suspended in fresh medium. Culture was passaged every two days.

3.6.1.16 Neural differentiation using all-*trans* retinoic acid

The procedure was basically according to the 4−/4+ induction method of Bain and co-workers with minor modifications (Bain et al. 1995). ES cell lines, D3 and E14TG2a, which have been propagated on PMEF were enzymatically segregated, replated for 45 minutes to deplete feeder cells and seeded in ES cell propagation medium on gelatinized 100-mm tissue culture dish for two days, were induced to form EB. ES cells were cultured for four days in the absence of RA followed by four days in

the presence of 0.5 mM RA. Control cultures were run in parallel for eight days without RA supplement.

Cultures were trypsinized and triturated to yield a uniform single cell suspension. One million cells in 10mL fibroblast medium without LIF and β-mercaptoethanol were cultured on a 100-mm diameter bacteriological Petri dish. Medium change was made every two days by gentle aspiration of spent medium into a sterile universal bottle. EB and cell aggregates were allowed to settle for three minutes. Supernatant was carefully removed. Fresh fibroblast medium without LIF and β-mercaptoethanol was replenished. Cell suspension was seeded in a new 100-mm diameter bacteriological Petri dish and incubated at 37°C in a humidified 5% CO_2-incubator. On day four, fresh fibroblast medium supplemented with 0.5 mM RA was used and changed every two days. Upon induction for eight days, cell aggregates were dissociated with 0.25% Trypsin containing 0.53 μM EDTA. Cell suspensions of 5×10^3 to 1×10^4 in fibroblast medium without LIF and β-mercaptoethanol were plated onto poly-L-ornithine- and laminin-coated coverslips and wells of 24-well tissue culture plate.

3.6.1.17 Stromal cells-derived inducing activity

Cultures of mitomycin C-inactivated feeder cells, C17.2, L cells and L-Wnt-3A, in six-well/12-well cell inserts on culture plates were washed with serum-free induction medium-I twice to remove residual serum. Trypsinized ES cells were replated onto gelatinized 100-mm tissue culture dish for 45 minutes to deplete feeder cells. One

hundred thousand single ES cells were seeded onto feeder cells in serum-free induction medium-I and cultured for eight days, followed by six days in serum-free induction medium-II (ITSFn medium; Okabe et al. 1996) and four days in serum-free induction medium-III, respectively. Medium was changed every alternative day.

For non-contact co-cultures, 1 x 10^6 and 1 x 10^5 single ES cells in 2.5 mL and 1.5 mL serum-free induction medium-I, respectively, were seeded onto six-well or 12-well culture plate. Inactivated feeder cells, which were established on 24 mm or 12 mm culture inserts a day prior to co-cultures, were washed twice, placed onto the corresponding culture plate and filled with 1.5 mL and 0.5 mL serum-free induction medium-I, respectively. Cultures were incubated at 37°C in a humidified 5% CO_2-incubator. Medium was changed every two days. On day four, culture inserts were replaced with new ones of confluent growth of freshly mitomycin C inactivated feeders. Co-culture was allowed to grow for four more days, followed by six days and four days in induction medium-II and in serum-free induction medium-III, respectively. Medium was changed every alternative day.

3.6.1.18 BrdU labeling of the cell products

Cultures of ES-derived cell products were labelled with BrdU a day before transplantation. The induction medium III were replaced by the Induction medium III supplemented with 10uM BrdU. Labeling was allowed for 24 hours (McDonald et al. 1999).

3.6.2 Molecular analysis

3.6.2.1 RNA extraction

Total RNA was isolated using RNeasy extraction kit (Qiagen) according to the manufacturer's protocol. Pellets of cells were vortexed and lysed in 400 μL Lysis Buffer RLT which containing guanidine isothiocyanate to protect RNA from degradation.

The mixture was applied to a silica-gel-membrane spin column and centrifuged at 8,000 g for 15 seconds. RNA was bound to the resin in the column while other metabolites eluted out. The spin column was cleaned with RW1 buffer by centrifuging at 8,000 g for 15 seconds followed by two washes in RPE buffer at 8,000 g and 20,000 g for 15 seconds and three minutes respectively. Total RNA was eluted with 30 μL RNase-free water treated with diethyl-pyrocarbonate (DEPC) into a micro-tube by spinning at 8,000 g for one minute and kept frozen at –80°C until further analysis.

3.6.2.2 RNA quantitation

RNA sample was diluted 100 folds in double deionized water. Absorbance values at 260 nm and 280 nm were measured against the buffer blank using the spectrophotometer. The reading at 260 nm allowed the calculation of the concentration of nucleic acid in the sample. An optical density (OD) of one corresponds to

approximately 40ng/μL for RNA. The ratio between the absorbance values at 260 nm and 280 nm gives an estimate for the purity of nucleic acid. Pure preparations of RNA had a ratio 1.6 to 2.1.

3.6.2.3 Reverse Transcription of the First Strand complementary DNA

Complementary DNA (cDNA) were transcribed from two μg total RNA with 100 ng random hexamers and 200 U Superscript reverse transcriptase in 20 μL reverse-transcription reaction mix containing 50 mM Tris-HCl buffer pH 8.3 at room temperature, 75 mM potassium chloride, three mM magnesium chloride, 10 mM dithiotreitol, 500 μM dNTP, 20 U RNase-Inhibitor. RNA and random hexamers were first incubated at 70°C for 10 minutes in a thermal cycler and quickly chilled on ice. The thermo-profile of cDNA generation was 25°C for 10 minutes, 42°C for 60 minutes, 95°C for five minutes and ended at 4°C.

3.6.2.4 Polymerase chain reaction

Five μL reverse-transcription products of the first strand cDNA from RNA transcripts were used for amplification of a specific gene sequence in a final 25-μL reaction volume. Table 3 shows details of the genes, primer-pairs, product sizes and thermoprofiles of the automated hot-start Polymerase chain reaction (PCR) at 94°C for five minutes to restore the activity of the inhibitor-bound polymerase. The reaction mix contained 20 mM Tris-HCl buffer pH 8.4, 50 mM potassium chloride, 1% Tween-20100

μM of each deoxynucleoside triphosphate (dNTP), six pmoles of each primer, 0.5U Platinum Taq DNA polymerase (Gibco BRL) and 1.5 – 2 mM magnesium chloride.

Table 3 Primer sequences of targeted gene and their thermal profiles

Gene	Primers Sequences	Products (bp)	Thermal Profiles and Reference
mEPO	Forward: 5' TCC TTG CTA CTG ATT CCT CTG G 3' Reverse: 5' AAG TAT CCG CTG TGA GTG TTC G 3'	451	94°C, 30sec;57°C, 30sec; 72°C, 1min; x 40 (Yamaguchi-Yamada et al. 2004)
mGDNF	Forward: 5′ CTG ACC AGT TTG ATG ACG TC 3′ Reverse: 5′ TCT AAA AAC GAC AGG TCG TC 3′	403	95°C, 1min; 60°C, 1min; 72°C, 2mins; x30 (Asaumi et al. 2000)
mGFAP	Forward: 5' AAC AAC CTG GCT GCG TAT AG 3' Reverse: 5' CGC TCT AGG GAC TCG TTC GT 3'	427	94°C, 30sec; 65°C, 45sec; 72°C, 1min; x 40 (Nicholson et al. 2001)
mIGF-1	Forward: 5′ TCG TCT TCA CAC CTC TTC TAC CTG 3′ Reverse: 5′ CTT CTG AGT CTT GGG CAT GTC AGT 3′	321	94°C, 1min; 55°C, 1min; 72°C, 1min; x 30 (Li et al. 2005)
mIGF-2	Forward: 5′ AGC GGC CTC CTT ACC CAA CT 3′ Reverse: 5′ GAA GTC GTC CGG AAG TAC GG 3′	345	95°C, 1min; 60°C, 1min; 72°C, 2mins; x33 (Asaumi et al. 2000)

Gene	Primers Sequences	Products (bp)	Thermal Profiles and Reference
mNestin	Forward: 5' GGA GTG TCG CTT AGA GGT GC 3' Reverse: 5' TCC AGA AAG CCA AGA GAA GC 3'	327	94°C, 30sec; 61°C, 1min; 72°C, 1min; x 35 (Lee et al. 2000)
mNGF	Forward: 5' GGA CTA AGC TTC AGC ATT CCC 3' Reverse: 5' ACA CTG TTG TTA ATG TTC ACC 3'	397	95°C, 1min; 57°C, 1min; 72°C, 2mins; 4x5 (Asaumi et al. 2000)
mNT-3	Forward: 5' GGT GAA CAA GGT GAT GTG ATG TCC ATC 3' Reverse: 5' GCT GCC CAC GTA ATC CTC CA 3'	391	95°C, 1min; 57°C, 1min; 72°C, 2mins; 4x5 (Asaumi et al. 2000)
mNurr1	Forward: 5' TGA AGA GAG CGG AGA AGG AGA TC 3' Reverse: 5' TCT GGA GTT AAG AAA TCG GAG CTG 3'	255	94°C, 30sec; 61°C, 1min; 72°C, 1min; x 35 (Lee et al. 2000)
mOct-4	Forward: 5' GGC GTT CTC TTT GGA AAG GTG TTC 3' Reverse: 5' CTC GAA CCA CAT CCT TCT CT 3'	312	95°C, 1min; 60°C, 1min; 72°C, 1mins; x25 (Ying et al. 2003)

Gene	Primers Sequences	Products (bp)	Thermal Profiles and Reference
mPAX-6	Forward: 5' TGC CCT TCC ATC TTT GCT TG 3' Reverse: 5' TCT GCC CGT TCA ACA TCC TTA G 3'	178	95°C, 1min; 54°C, 1min; 72°C, 2mins; x33 (Pachernik et al. 2002)
mVEGF	Forward: 5' GCG GGC TGC CTC GCA GTC 3' Reverse: 5' TCA CCG CCT TGG CTT GTC AC 3'	716, 644, 512	94°C, 1min; 65°C, 1min; 72°C, 1.5mins; x30 (Marti et al. 1998)
mWnt-3A	Forward: 5' GGA ATG GTC TCT CGG GAG TTT G 3' Reverse: 5' TTC GGG GTT AGG TTC GCA GAA G 3'	372	94°C, 30sec; 60C, 30sec; 72°C, 45sec; x 30 (Derfoul et al. 2004)
mα-fetoprotein	Forward: 5' ATG TAT GCC CCA GCC ATT CTG TCC 3' Reverse: 5' GAG ATA AGC CTT CAG GTT TGA CGC 3'	466	95°C, 1min; 54°C, 1min; 72°C, 2mins; x29 (Pachernik et al. 2002)

3.6.2.5 RNA Integrity Check

RNA integrity was confirmed by amplifying the reverse-transcription product of the first strand cDNA from the ubiquitous mRNA of Glyseraldehyde-3-phosphate dehydrogenase (GAPDH). The amplified gene product of 452 bp was obtained with primers, ACC ACA GTC CAT GCC ATC AC (sense) and TCC ACC ACC CTG TTG CTG TA (anti-sense), upon completion of the 30-cycle amplification of denaturation at 94°C, annealing at 60°C and synthesis at 72°C for 30, 30, 72 seconds each. Magnesium chloride 1.5 mM was used.

3.6.2.6 Electrophoresis and visualization of gene products

PCR products and the molecular marker of 100-bp DNA ladder were separated and run in 1-2% agarose gel by electrophoresis, stained with ethidium bromide and visualized by using the UV transilluminator. Gel images were captured by either the direct screen instant camera or the electrophoresis documentation and analysis system. Results were considered valid if readouts of all replicate analyses were in concordance.

3.6.3 Immunofluoresent staining

Upon completion of fixation for 20 minutes in 4% paraformaldehyde (PFA) at room temperature, cells were permeabilized for five minutes in 0.3% Tritron-X in PBS. Sections were washed thrice in PBS, blocked with 10% normal goat serum (NGS) in

PBS at room temperature for 10 minutes, followed by washing thrice with PBS containing 1% NGS. Cells were then incubated overnight at 4°C with optimally diluted primary antibodies of specificities stipulated in Table 4. After washing thrice with PBS, cells were detected by incubating with the pertaining secondary antibodies diluted 100 folds in 1% NGS in PBS for 30 minutes at room temperature. They were namely FITC-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-mouse IgG (H+L) and TRITC-conjugated goat anti-mouse IgG (H+L). Slides of sections were washed thrice in PBS and mounted with anti-fade aqueous mountant containing the fluorescent dye of either propidium iodide (PI) or 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) as counter-stain. Sections were examined under a fluorescent microscope.

Table 4 Specificities and dilutions of primary monoclonal antibodies used in immunofluoresent staining.

Phenotype	Specificity	Isotype	Dilution Factor
ES cell	Stage-Specific Embryonic antigen-1	IgM	100
	Octamer-binding transcription factor-4	IgG	100
	Tumor rejecting antigen 1-81	IgM	100
Neural Stem Cell	Neuroepithelial stem cell intermediate filament	IgG	400
Neuron	beta-tubulin class III	IgG	1000
	Microtubule-associated Protein 2	IgG	100
Astrocyte	Glial Fibrillary Acidic Protein	IgG	50
DA Neuron	Tyrosine Hydroxylase	IgG	500
Oligodendrocyte	Myelin Basic Protein	IgG	200

3.6.4 *In-vivo* studies

3.6.4.1 Induction of cerebral ischaemia in mice

Male ICR mice of mean age 12 weeks and body weight of 25 to 35 g were obtained from Laboratory Animal Services Centre of the Chinese University of Hong Kong. Global cerebral ischaemia was induced by transient BCCAO of male ICR mice having anesthetized by Intraperitoneal injection of 300 μL of 1% ketamine and 0.1% xylazine in normal saline. A cervical incision was sterilely made on the midline of the ventral neck. Bilateral common carotid arteries in the neck were exposed and occluded with micro-aneurysm clips for 15 minutes. Thereafter clips were released to allow reperfusion. The skin incision was sutured and the mice were allowed to gain access to food and water ad libitum in micro-isolator cages.

3.6.4.2 Transplantation

One week post-induction of cerebral ischaemia, tested animals were anesthetized sedated and fixed to the stereotaxic frame. The stereostaxic co-ordinates were one mm anterior and 2 mm lateral to the bregma. The Hamilton syringe was inserted four mm into the brain parenchyma from the surface. ES cell-derived products were mechanically dissociated into single cell suspension in DPBS and adjusted to approximately 1 x 10^5/μL. Five μL cell suspension was slowly injected into mice at a rate of one μL per minute. In the sham groups, five μL DPBS were injected. The needle

was slowly drawn after 10 minutes. The hole was cemented and the skin incision was sutured.

3.6.4.3 Assessment of learning ability and memory

Test and control animals were assessed by using the water maze system which is intended to evaluate the learning ability and memory of mice. Basically the maze was so constructed with four cue de sacs diverged from the go-through route from the starting point to the finishing exit. The system was filled with water to a depth of 15 cm to wash out likely body scent which might be regarded as an indicator of way-out by mice in subsequent test. The water temperature was maintained at around 26°C. Mice of interest were daily trained for three days prior to formal assessments on the two consecutive days. The time taken to complete the journey and number of errors committed by entering and returning to the dead ends were reckoned. Comparisons were made among groups of normal mice, ischaemic mice and ischaemic mice having undergone cellular therapy of ES cell-derived product.

3.6.5 Histological analysis

3.6.5.1 Animal sacrifice for brain harvest

Upon completion of *in-vivo* monitoring, test and control animals were anesthetized. The thoraxes were cut open and animals were trans-cardiacally perfused

with 30 mL normal saline. Heads were cut away from the bodies and the whole brains were removed quickly and carefully from the skulls. They were then transferred to liquid nitrogen or 4% PFA.

3.6.5.2 Cryosectioning

Freshly isolated brains were kept quickly frozen in liquid nitrogen. Blocks of tissues embedded in the cryo-mountant OCT were equilibrated for five to ten minutes in the microtome. Cryostat sections of 10 to 20 μm in thickness were cut and mounted onto superfrost plus slides or slides coated with 3-Aminopropyl triethoxy-silane (APES; Cat. A3648, Sigma). Slides were kept at -80°C until staining.

3.6.5.3 Paraffin sectioning

Freshly removed brains of the tested animals were fixed for three days in 30 mL 4% PFA. They were cut into appropriate sizes and placed in embedding cassettes. The tissues were dehydrated sequentially in up-grading concentrations of 70%, 80%, 95% ethanol for one hour twice and absolute ethanol for one hour thrice. Brains were then subjected to xylene for one hour thrice, 56°C paraffin wax for 1.5 hours twice, before final paraffin embedding.

Paraffin blocks of tissues, of either coronal or lateral plan, were cooled down to -20°C for 20 minutes before trimming. Sections of five to 10 μm in thickness were cut

by using a microtome. They were spread onto surface of water at 45°C. Well spread section was transferred onto APES-treated glass slides, air dried and then baked in a 56°C-oven overnight. Slides were kept at room temperature until use.

3.6.5.4 Haematoxylin and eosin staining

Slides of frozen sections were fixed in 10% formalin for 1 minute before staining. Paraffin-embedded sections were dewaxed in xylene for five minutes twice. They were then re-hydrated in absolute to down-grading 95% and 70% alcohol for five minutes twice, two minutes each, respectively. Slides of sections were eventually washed in distilled water and stained in Harris hematoxylin solution. After 10 minutes, the sections were washed in running tap water for two minutes and rinsed in Scotts' tap water for approximately one minute. The sections were washed in running tap water again for two minutes, counters-stained in eosin-phloxine solution for three minutes. Having rinsed in running water, stained sections were dehydrated in up-grading concentration of 70% and 95% alcohol for two minutes each and absolute ethanol for five minutes twice. Sections were then cleared in xylene for five minutes twice and mounted using appropriate covers-lips and xylene-based mountant.

3.7 Data analysis

Non-parametric Mann-Whitney test was used to analyze continuous variables of cell nestin-positive colonies. ANOVA was used to examine the incidence of errors committed, and time intervals for completion of journey among normal mice, ischaemic mice and ischaemic mice having undergone cellular therapy of ES cell-derived product. Student's T-test was used to analyze the continuous variable of viability, cell number and SSEA-1 positivity. Differences between groups were regarded as significant if $p \leq 0.05$.

Chapter 4 Results

4.1 ES cell maintenance

4.1.1 Serum effect

Upon thawing, ES cells D3 which were maintained in ES cell propagation medium for two days and were then passaged. ES cells of 3 x 10^5 were allowed to grow in ES cell propagation medium supplemented with three different foetal calf sera at 15% (volume/volume) on gelatinized dishes pre-established with confluent growth of mitotically inactivated STO. Table 5 shows the numbers of ES cell colonies derived from three random fields of 10x objective in three separate experiments. The mean number ± one standard deviation of ES cell colonies in cultures with Hyclone serum supplement was 9 ± 1, being significantly fewer than 22 ± 6 ($p=0.0425$) and 30 ± 2 ($p=0.005$) derived from Gibco and ES-tested serum, respectively. In terms of the number of segregated ES cells and cell viability, cultures supplemented with Hyclone serum gave the lowest yields (Figure 3). Besides, cells with relatively abundant and irregular cytoplasm were noted in cultures supplemented with Hyclone serum as compared to small discoid mononuclear cells with a rim of cytoplasm derived from culture supplemented with ES-tested serum (Figure 4). There was also an increase in the numbers of differentiated cells without the expression of the ES cell marker, SSEA-1 in culture supplemented with Hyclone serum after two passages (Figure 5).

Table 5 Numbers of ES cell colonies derived from three random fields of 10x objective in three separate experiments on ES cell D3 in culture medium supplemented with three foetal calf sera.

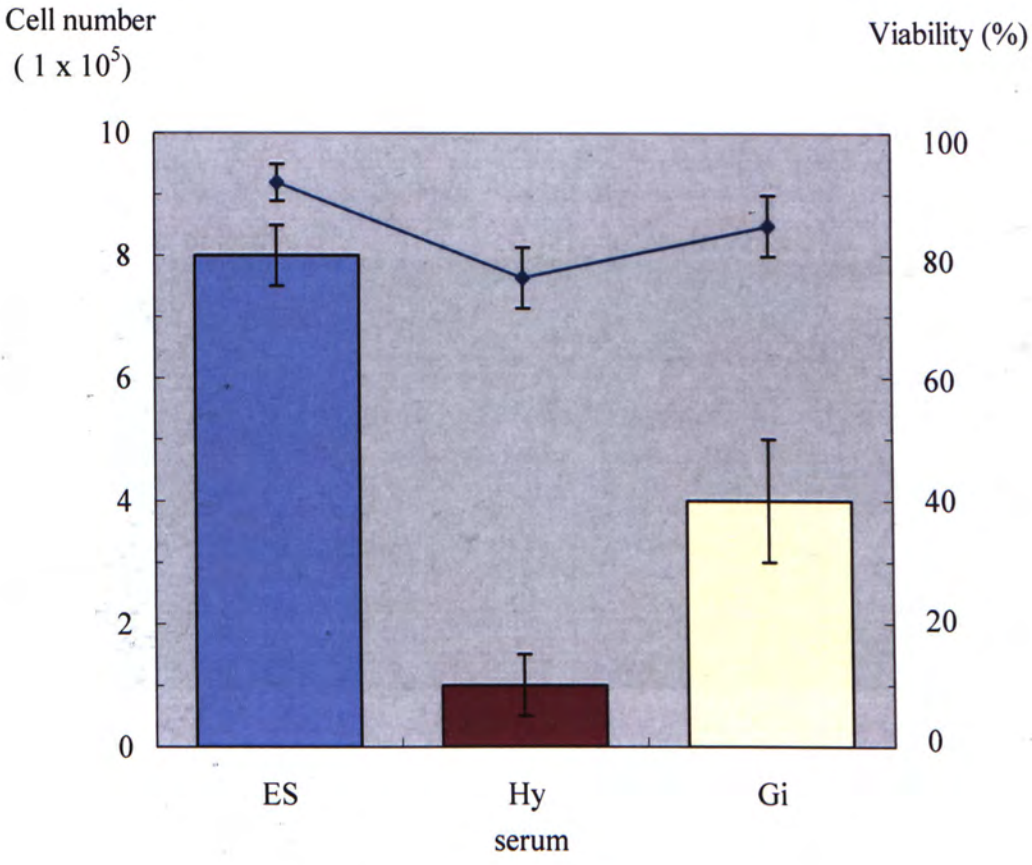
	Number of colonies per 3 x 10^5 ES Cells D3		
Experiment	ES	Hy	Gi
I	28	8	28
II	32	10	18
III	30	10	19

ES: ES tested serum

Hy: Hyclone

Gi: Gibco

Figure 3 Total cell numbers (left axis) and cell viability (right axis) derived from three separate experiments on ES cell D3 culture supplemented with three different foetal calf serum



ES: ES-tested serum

Hy: Hyclone

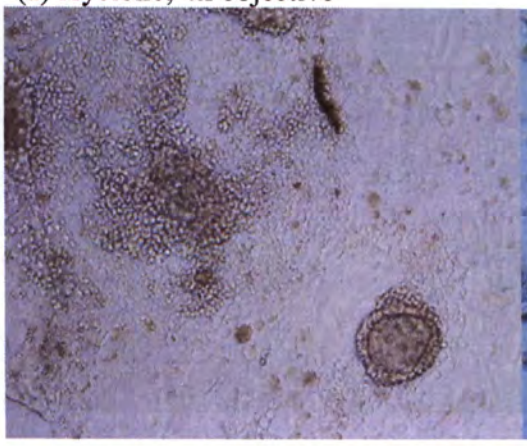
Gi: Gibco

Colour bars: Cell numbers in 10^5

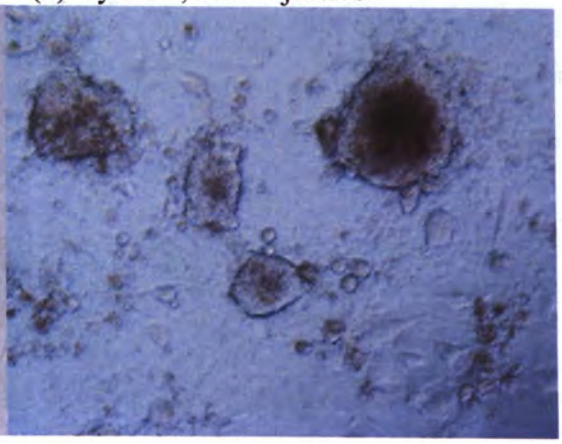
Blue line: Cell viability

Figure 4 Morphology of ES cell D3 maintained in cultures supplemented with two different foetal calf sera. (a) ES cells showed signs of differentiation in the cultures with Hyclone serum. Some ES colonies were flattened and surrounded by a circle-like boundary, and appeared as individual cells rather than as syncial mass. (b) Some differentiated ES colonies had necrotic centers with defined boundaries and edges appear dark and irregular. (c) Undifferentiated ES colonies appeared as syncial mass.

(a) Hyclone; 4x objective



(b) Hyclone; 10x objective



(c) ES-tested; 20x objective

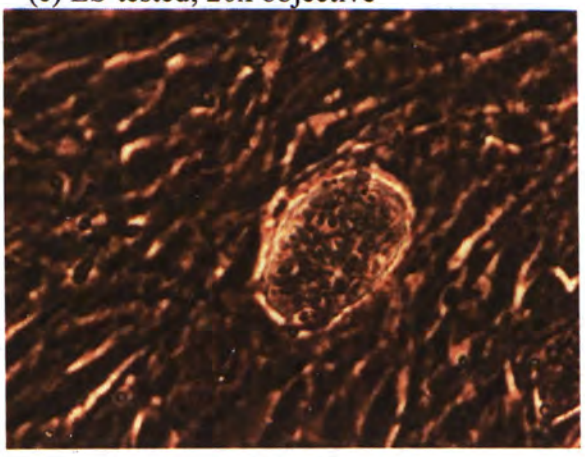
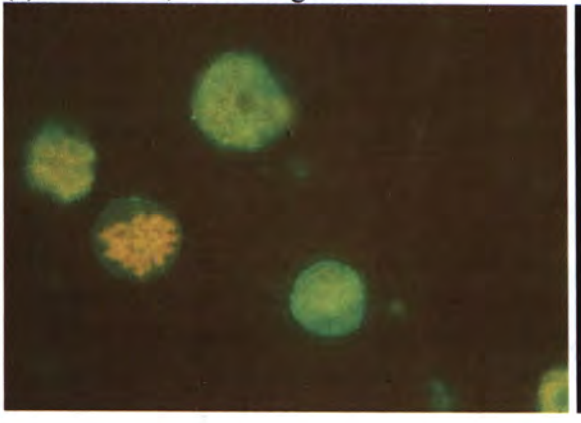
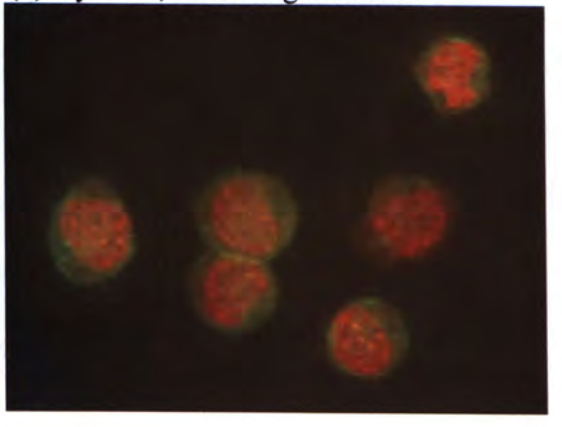


Figure 5 Immuno-staining of SSEA-1 on ES cell D3 maintained in cultures supplemented with three different foetal calf sera after two passages. ES cells harvested from the three different cultures showed SSEA-1 expression (a-c; green fluorescent), with cells from ES-tested serum (a) showing a stronger intensity than that of Hyclone serum (b). The results also show that undifferentiated mouse ES cells have a large nucleus to cytoplasm ratio as shown in (c). These slides were counter-stained with PI to show the nuclear structures.

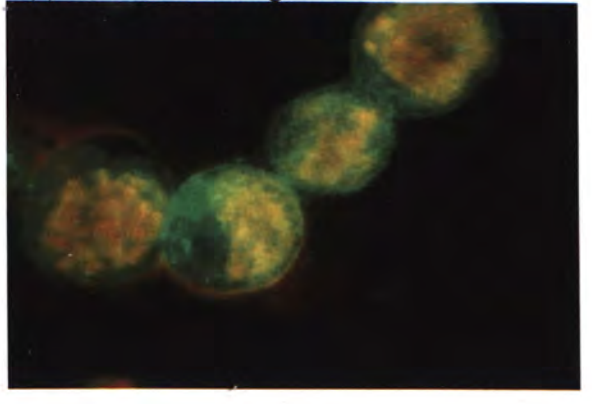
(a) ES-tested; 150x magnification



(b) Hyclone; 150x magnification



(c) Gibco; 200x magnification



Data of this evaluation suggested that there might be less growth-promoting factors to ES cells in Hyclone serum. There were also likely toxic substances and differentiating agents to inhibit propagation and enhance differentiation of ES cells, respectively. In subsequent downstream analyses employing serum-based medium, ES-tested serum was used.

4.1.2 Feeder effect

Primary cultures and established cell lines of mouse embryonic fibroblasts producing LIF play a crucial in the propagation of mouse ES cells *in-vitro* (O'Shea 2004). Table 6 shows the numbers of SSEA-1^+ ES colonies D3 maintained on chamber slides pre-established with and without PMEF in three separate experiments. The percentage of SSEA-1^+ colonies derived from PMEF support was significantly higher than that without feeder (91.0% ± 3.6% vs. 80.0% ± 4.4%, p=0.032). In terms of the number of SSEA-1^+ ES colonies, cultures with and without PMEF yielded a comparable number of ES cell colonies (Figure 6). However, the percentage of SSEA-1^+ colonies were higher in cultures with than without PMEF support (p=0.030). Data suggest that PMEF could help maintain the ES cell stemness from differentiation.

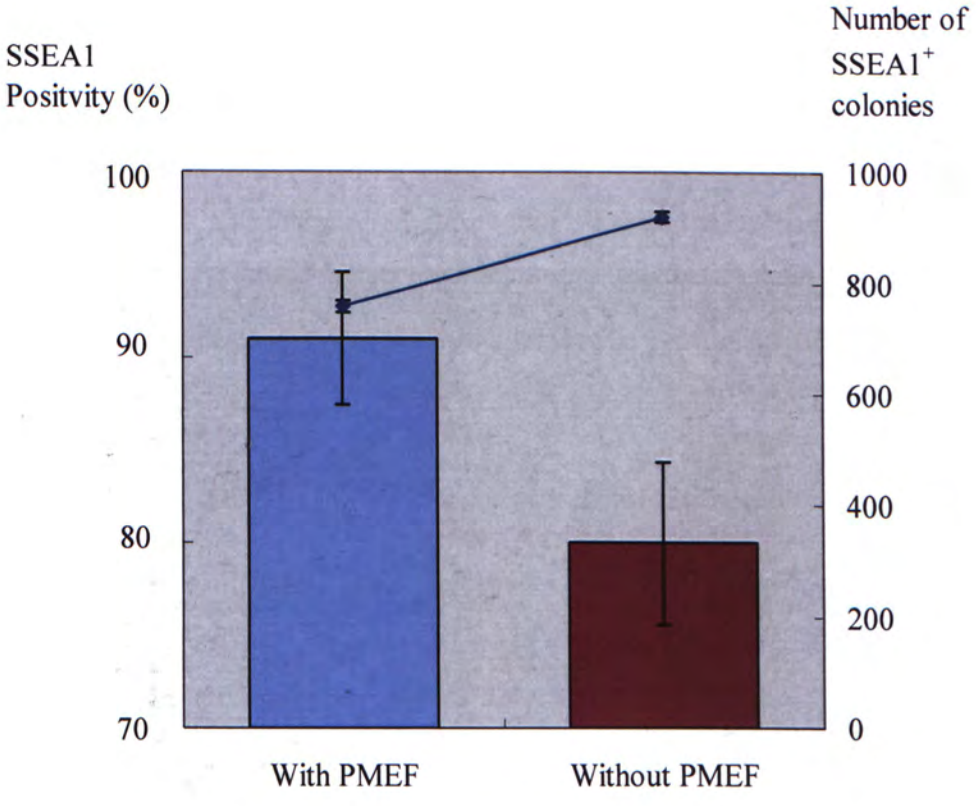
The efficacy of mouse embryonic fibroblasts derived from either primary cultures or established cell line in ES cell maintenance was also evaluated. Figure 7 shows the total numbers of ES cells D3 and the extents of cell viability in two consequent passages in three separate experiments using chamber slides. Both PMEF

and STO supported the growth of undifferentiated ES cells D3 and the extents of cell viability were above 95% ± 3%. From the average of three separate experiment, ES cells D3 maintained on PMEF propagated at significantly higher rates as compared to those derived from STO in two consequent passages (first passage in two days: fold increase of cell numbers in PMEF vs. STO: 4.7 ± 0.29 vs. 3.0 ± 0.31, p=0.001; second passage in one day: 5.5 ± 0.28 vs. 3.8 ± 0.14, p=0.012). The stemness of ES cells D3 maintained on PMEF and STO was also evaluated. A comparatively higher number of SSEA-1^+ cells was noted in ES cell cultures maintained on PMEF (PMEF vs. STO: 92.1 ± 4.2% vs. 83.3 ± 2.9%, p=0.029) as shown in Figure 8. Having replated onto tissue culture dishes for 45 minutes to get rid of PMEF, enriched ES cells D3 derived from the second passage were immuno-stained for SSEA-1 (Figure 9). Data on the cell number, viability and SSEA-1 immuno-positivity suggested that PMEF was superior to the mouse embryonic fibroblast cell line STO in the propagation and maintenance of ES cells.

Table 6 Number and percentage of SSEA-1$^+$ ES colonies in three separate maintenance cultures of ES cell D3 with and without feeder support of primary mouse embryonic fibroblasts in chamber slides.

	Mean number and Percentage of SSEA1$^+$ ES colonies	
Experiment	With PMEF	Without PMEF
I	252 (87)	317 (78)
II	242 (94)	304 (85)
III	264 (92)	299 (77)

Figure 6 Comparisons of SSEA-1 positivity and number of colonies of ES cells D3 propagated and maintained with or without PMEF.



Colored bars: SSEA-1 positivity

Blue line: colony number

Figure 7 Total number of ES cells D3 and cell viability in two consecutive passages derived from PMEF and STO support (as shown) on chamber slides in three separate experiments.

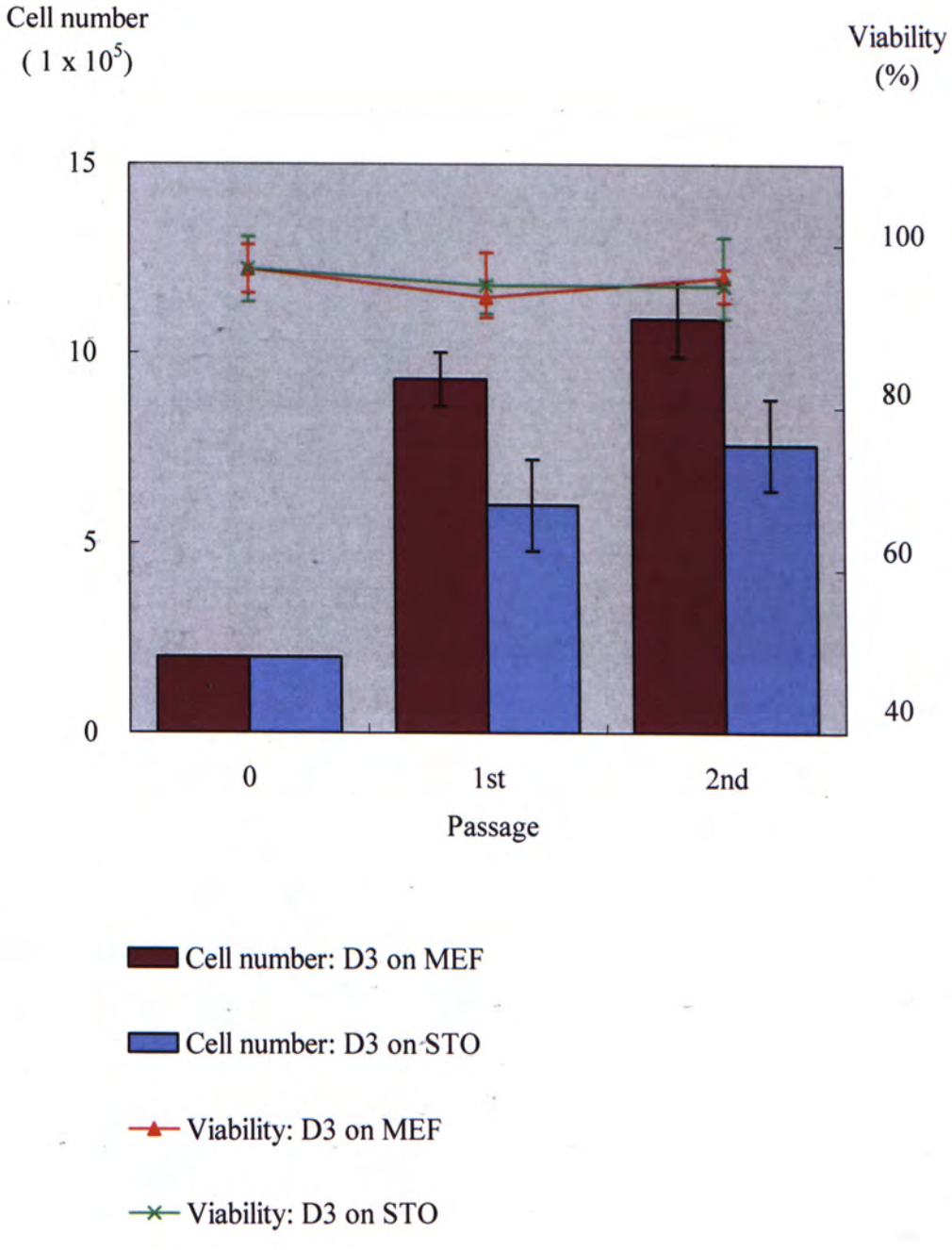
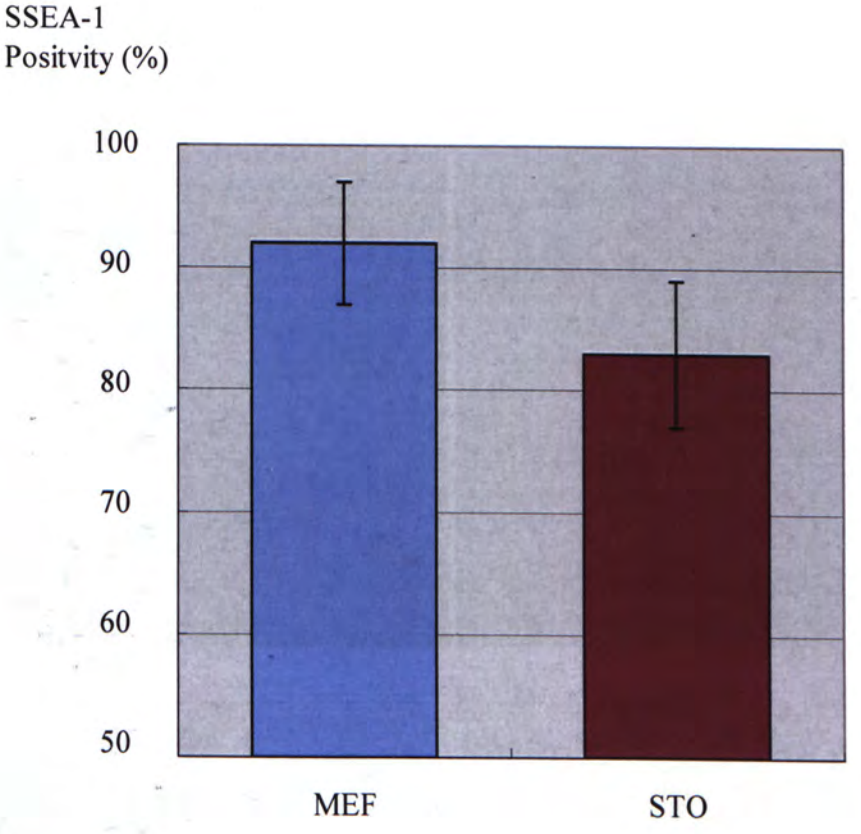


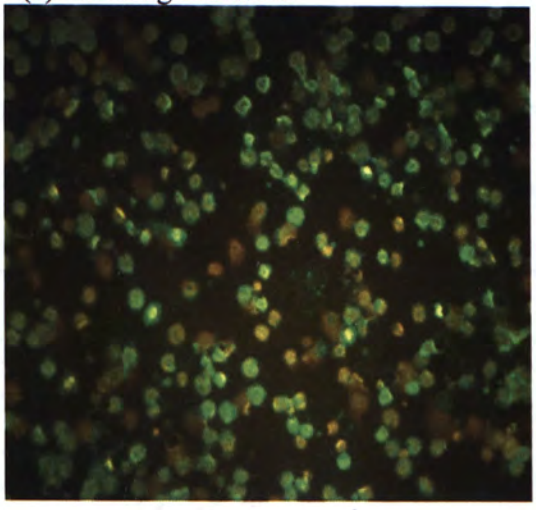
Figure 8 The extent of stemness in terms of SSEA-1 immuno-positivity of ES cells D3 have undergone two passages on PMEF and STO.



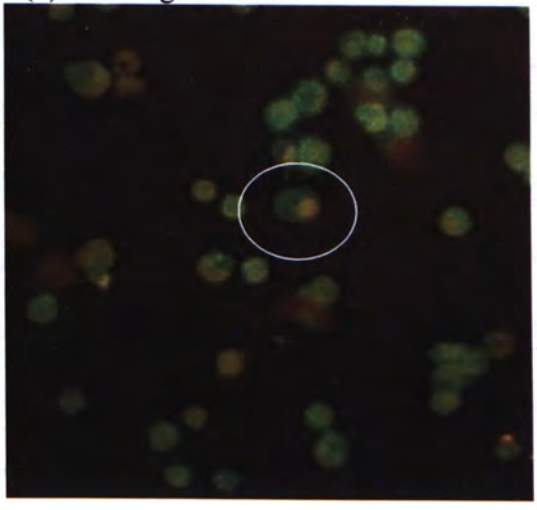
In the propagation of ES cells using feeder cell support, there was a nominal number of residual inactivated feeder cells, despite the ES cell product was re-plated onto tissue culture dishes for 45 minutes. They could easily be distinguished from undifferentiated ES cells under microscope.

Figure 9 SSEA-1 immuno-reactivity of ES cells D3 maintained on PMEF and passaged twice. Almost all D3 were SSEA-1^{+}. The majority of PMEF (SSEA-1^{-}) were removed by their adherent property. A very small portion of differentiated ES cells would still show SSEA-1-positivity, which was characterized by a relatively small nucleus (white circle in (b)), when compared with undifferentiated ES cells'.

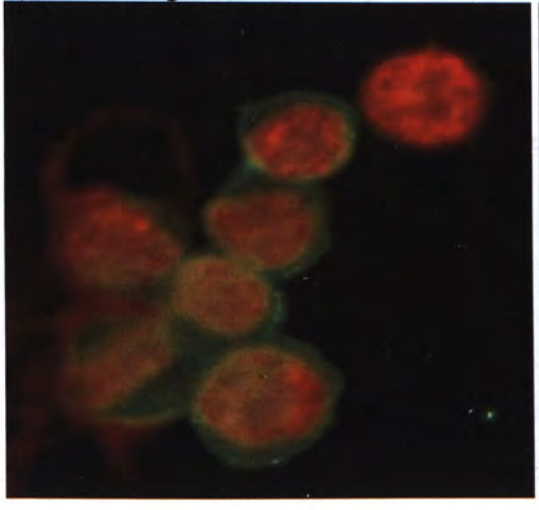
(a) 40x magnification



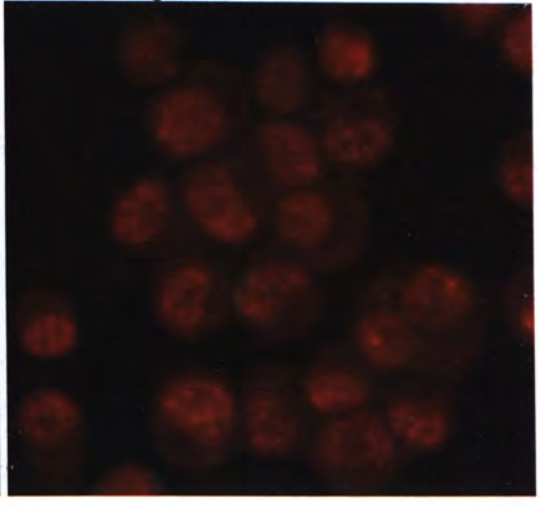
(b) 60x magnification



(c) 200x magnification



(d) STO negative control

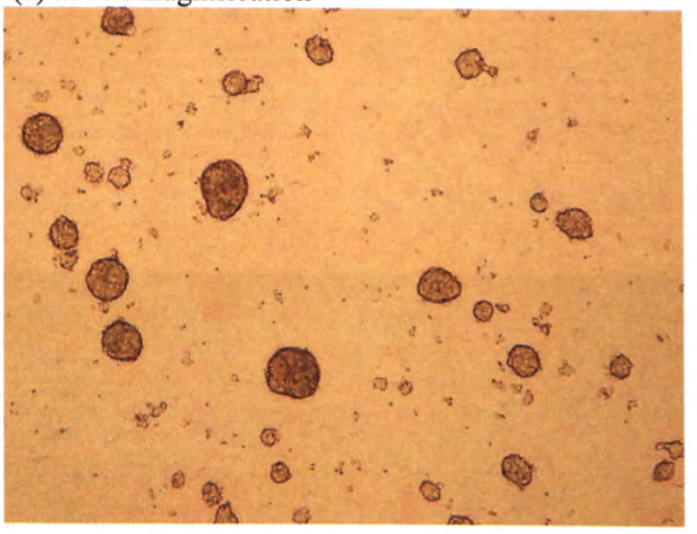


4.1.3 Serum-free and feeder-free condition

Pertaining to the constraint of quality serum and synchronization of feeder cell culture in the propagation of ES cells, a protocol employing serum-free and feeder-free culture condition would be advantageous. ES cells D3 were seeded separately onto low binding flasks, ultra-low adherent plates and bacterial Petri dishes, of serum-free culture medium at 1 x 10^5 per mL. Non-adherent cell clusters of variable sizes and irregular contour were noted after two days, which accounted for more than 90% of the culture (Figure 10). Some have sprouting processes indicating signs of differentiation. Cultures were split and propagated in fresh medium for further two days. Figure 11 shows data of three separate experiments. The SSEA-1 reactivity of the first passage on day 2 was 82.6% ± 5.7%, whereas SSEA-1^+ cells of the second passage on day 4 decreased significantly to 63.7% ± 5.0% ($p=0.001$), which were far less than those maintained in serum-based medium with PMEF support ($p=0.009$).

Figure 10 Non-adherent cell clusters of ES cells D3. Variable sizes and irregular contour were observed from serum-free and feeder-free cultures for two days. Process sprouting was evident in some cell clusters.

(a) at 40x magnification



(b) at 60x magnification

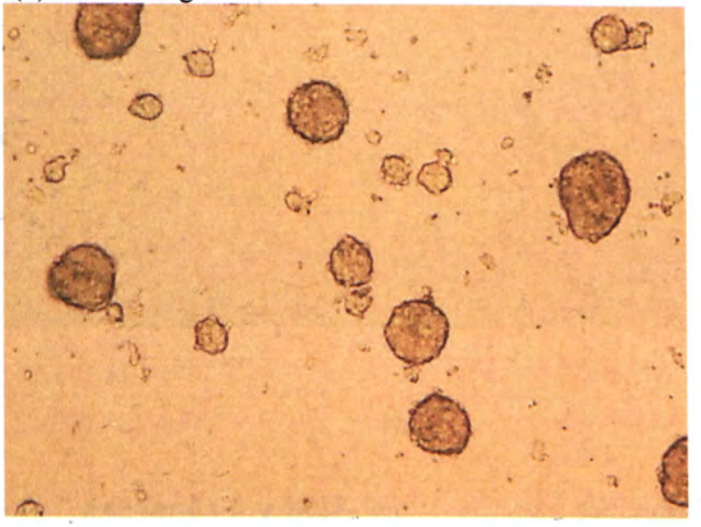
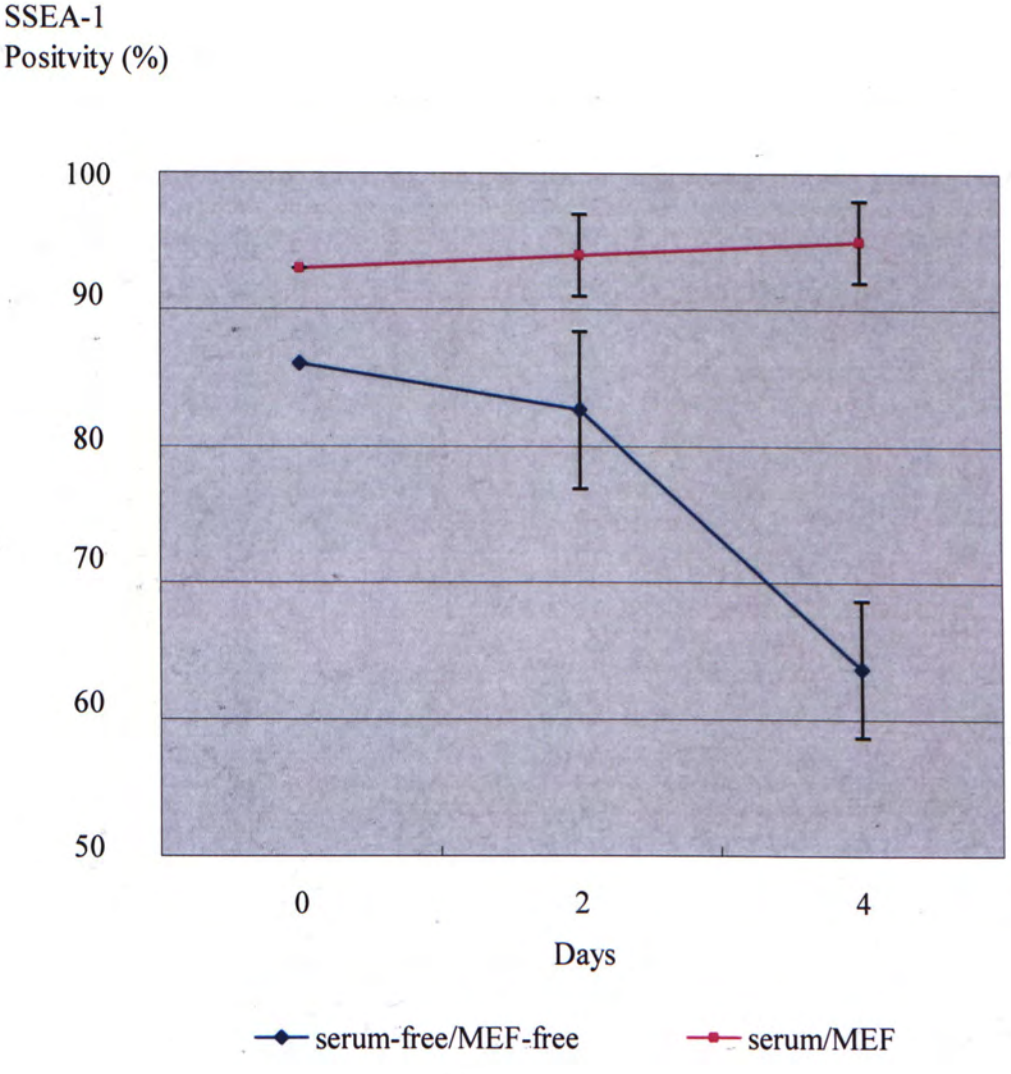


Figure 11 Immuno-reactivity of SSEA-1 in ES cells D3 propagated in serum-free medium without feeder support on non-adherent culture wares. Data were derived from two consecutive passages in three separate experiments.

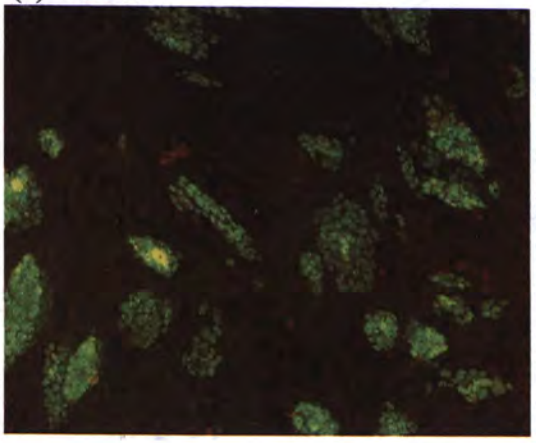


4.1.4 Overall effect

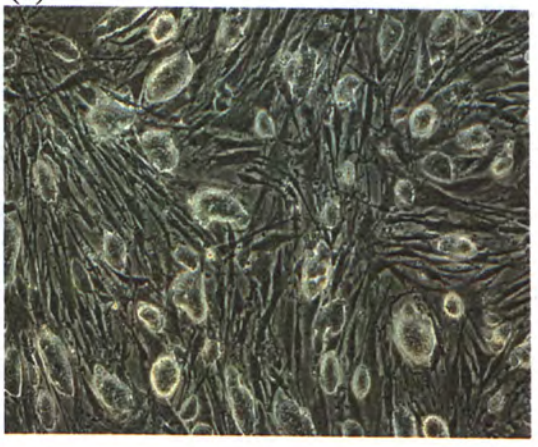
The optimized culture condition on ES cells D3 was attested on ES cell line, E14TG2a. The propagation medium supplemented ES-tested serum in conjunction with PMEF feeder support worked equally well in propagating E14TG2a without any sacrifice of the stemness. Figure 12 shows that they were morphologically undifferentiated and immunologically positive to SSEA-1, Oct-4 and Tra1-81, which are markers of undifferentiated ES cells, suggesting the applicability in the propagation and maintenance of ES cells.

Figure 12 Immuno-staining of Tra1-81, Oct-4 in ES cells D3 and E14TG2a maintained on PMEF in serum-based propagation medium for two days. ES cells formed colonies on PMEF, and they are immuno-positive with Tra1-81 and Oct-4 (green fluorescent counter-stained with PI). (a-b) E14TG2a on PMEF, (c-d) D3 on PMEF. (a) and (c) were observed under fluorescent microscopy. (b) and (d) were observed under phase-contrast microscopy and background showed PMEF.

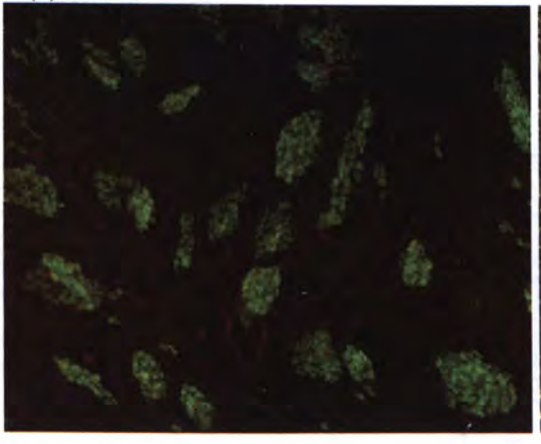
(a) Tra1-81



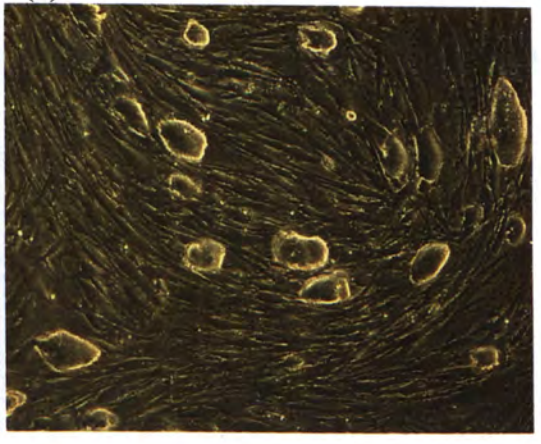
(b)



(c) Oct-4



(d)



4.2 ES cell Induction

4.2.1 Retinoic acid

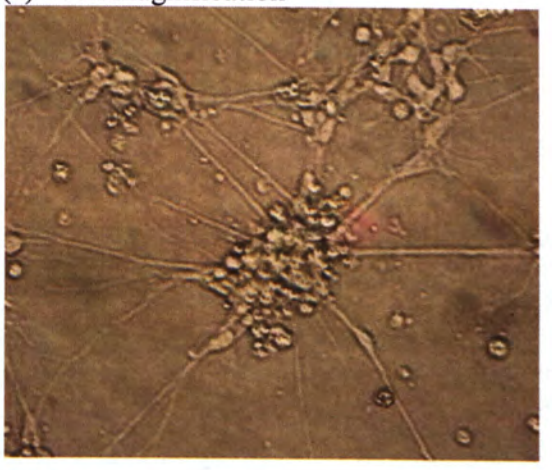
Table 7 shows viabilities of three separate experiments employing 0.5 mM RA in the differentiation of ES cells D3. Upon induction for eight days, the mean proportion ± one standard deviation of cell viability of trypsinized cells derived from RA-mediated differentiation culture was 81.5% ± 2.5%, being significantly lower than 88.3% ± 2.5% of the controls ($p=0.046$). Besides, RA-induced cells displayed morphologies of neural cell lineage with sprouting processes and networks with neighbouring cell foci (Figure 13). Immunohistochemistry demonstrated the immature neuronal marker of TuJ-III, NSC marker of nestin, astroglial marker of GFAP and oligodendroglial marker of MBP (Figure 14). The mean percentage of immuno-positive cells were shown in the Table 8. Among TuJ-III$^+$ cells, the majority gave little to no immuno-reactivity with, the marker of matured neurons antibodies, MAP-2, suggesting that the presence of immature neurons.

Table 7 Cell viability of ES cell-derived EB in three separate experiments.

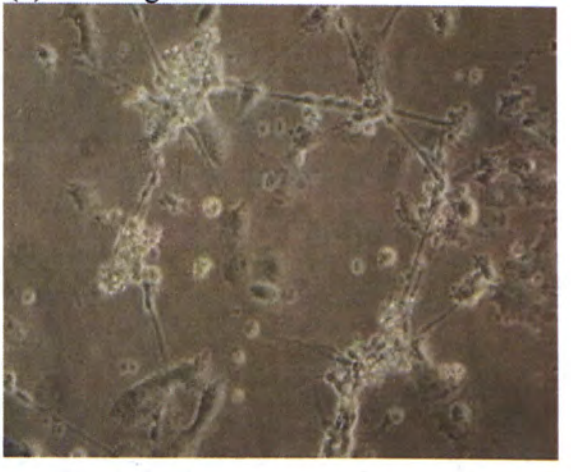
	Cell Viability in Percentage	
Retinoic Acid	With	Without
I	78.8	88.0
II	82.0	86.0
III	83.7	91.0

Figure 13 Morphology of retinoic acid-induced neural cell lineage from D3 observed under phase contrast microscopy. They formed network with neighbouring cell foci.

(a) 100x magnification



(b) 60x magnification



(c) 40x magnification

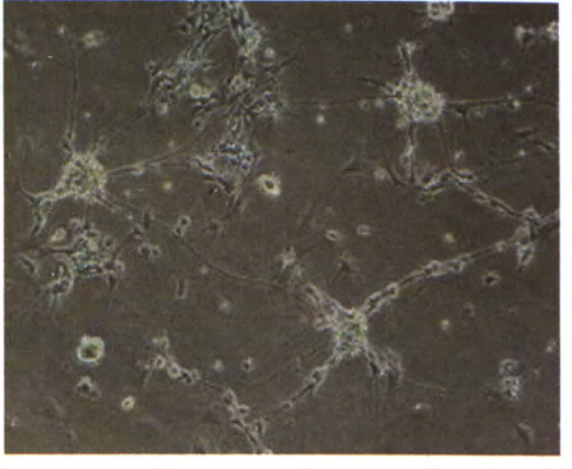


Figure 14 Immuno-reactivity of RA-induced cell product showing morphology of neural cell lineage. (a) neural stem cell marker nestin (green fluorescent), (b-d) immature neuronal marker TuJ-III (green fluorescent), at 100x magnification under fluorescence microscopy. TuJ-III^{+} formed network with neighbouring cells. These slides were counter-stained with PI to show the nuclear structure.

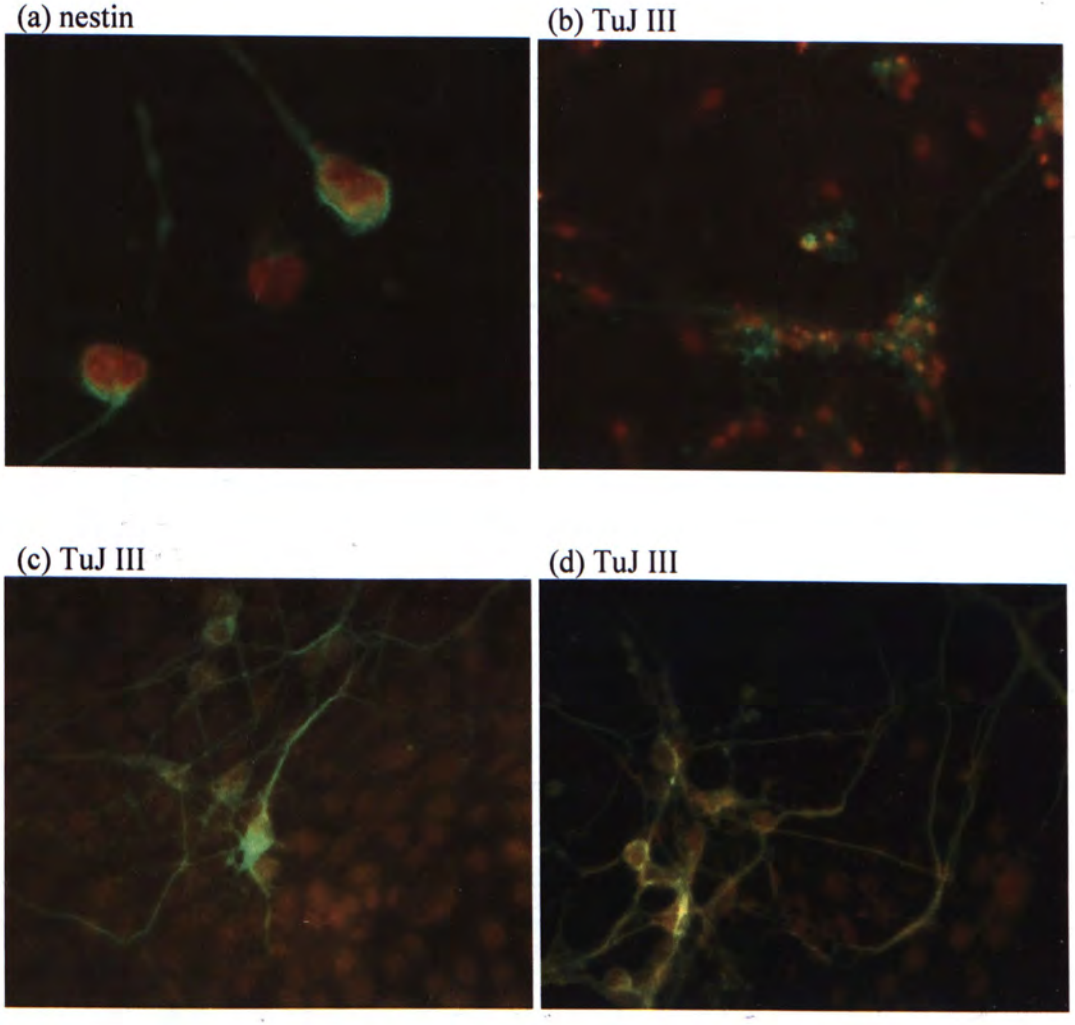


Table 8 Proportion of neural cell lineage commitment of RA-induced cell product.

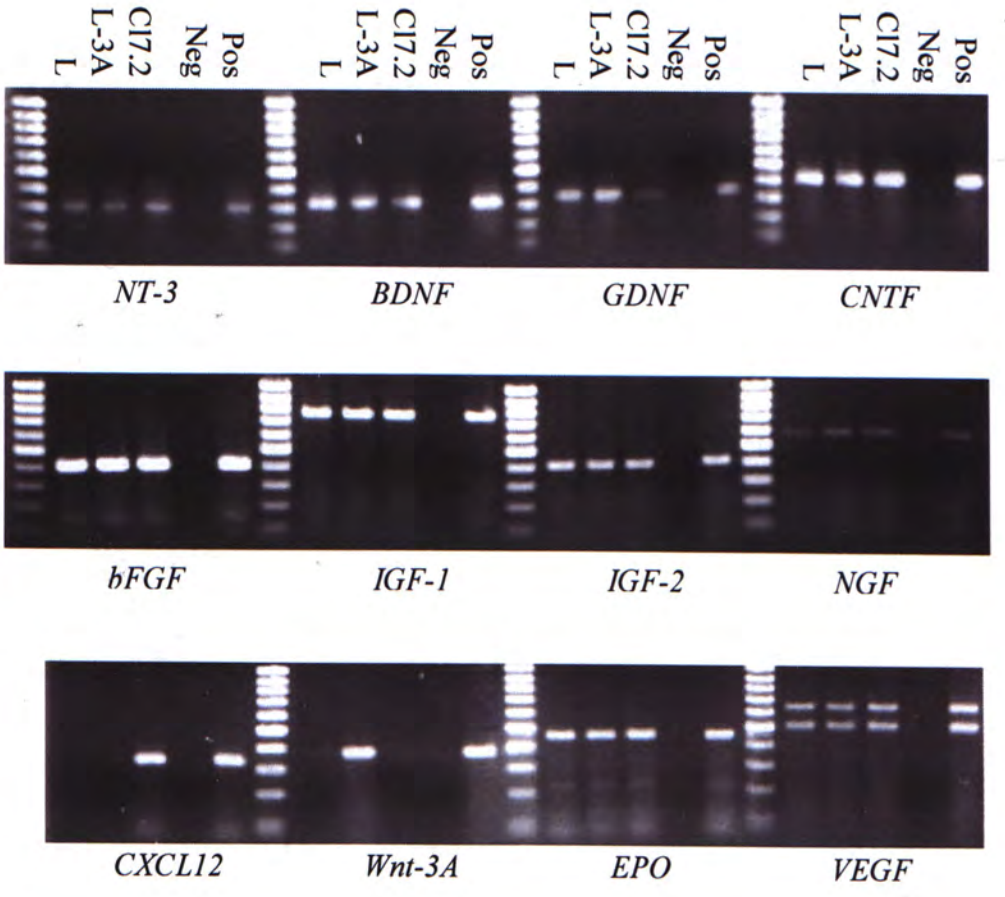
Marker (Lineage)	Percentage of Positivity	
	RA	Control
TuJ III (Neurons)	39	3
Nestin (Neural Stem Cells)	29.5	>1
GFAP (Astrocytes)	>1	>1
MBP (Oligodendrocytes)	>1	>1

4.2.2 Stromal cell-derived inducing activity

4.2.2.1 Molecular characterization of candidate stromal cells

The potential applicability of C17.2, L and L-Wnt-3A as feeder layers in exerting SDIA for ES cell derivation into neural cell lineage were examined by RT PCR for gene transcripts of neurotrophins, growth factors and chemokines. Figure 15 illustrates that C17.2, L and L-Wnt-3A cell lines expressed neurotrophins of *BDNF*, *NT-3, NGF, GDNF, CNTF* and *IGF-1 & 2*. The feeders also expressed *bFGF*, which is well-known growth–promoting factor for NSC. Besides, neural inducing factors of *VEGF* and *EPO*, which were noted to promote neural differentiation, were also detected in these feeder cells. Chemokine (C-X-C motif) *CXCL-12* and *Wnt-3A* were only detected in the neural precursor cell line C17.2 and the virally transfected L-Wnt-3A cells, respectively. Data derived from molecular analyses suggested that these cell lines may be candidate stromas to mediate SDIA for neural differentiation of ES cells.

Figure 15 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products namely, *NT-3*, *BDNF*, *GDNF*, *CNTF*, *IGF-1*, *IGF-2*, *NGF*, *bFGF*, *EPO*, *VEGF*, *CXCL-12* and *Wnt-3A*, derived from neural precursor cells C17.2, L cells and L-Wnt-3A cells.



Pos: positive control

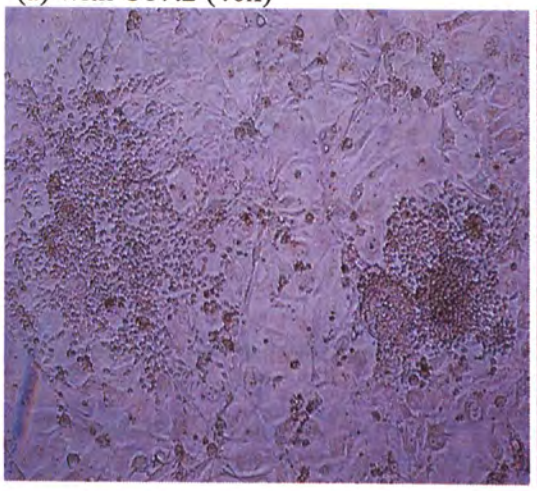
Neg: negative control

4.2.2.2 Direct contact co-culture

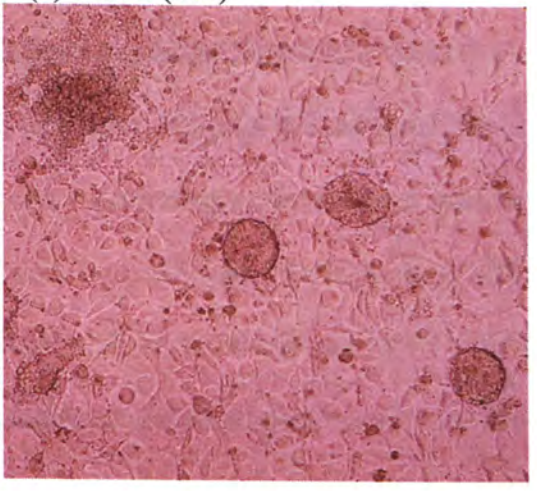
Discrete ES cells, D3 and E14TG2a, were seeded separately onto pre-established mitomycin C-inactivated feeders, C17.2, L, L-Wnt-3A and cultured for eight days. Some were differentiated and became morphologically indistinguishable from the feeders (Figure 16). Despite attempts of extensive inactivation to the stromal cells using prolonged mitomycin C treatment or γ-irradiation, isolation of SDIA-induced cell products were difficult.

Figure 16 Nucleation of growing cell foci of ES cells D3 seeded onto stromas (co-cultured for eight days). (a) D3 with C17.2 and (b) D3 with L under phase contrast microscopy, (c) D3 with C17.2 and (d) with L cells.

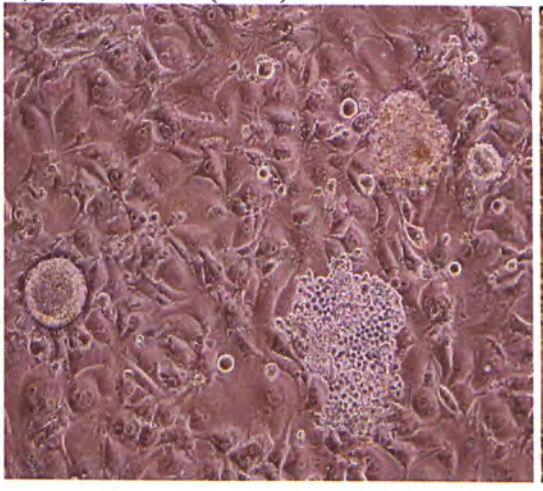
(a) with C17.2 (40x)



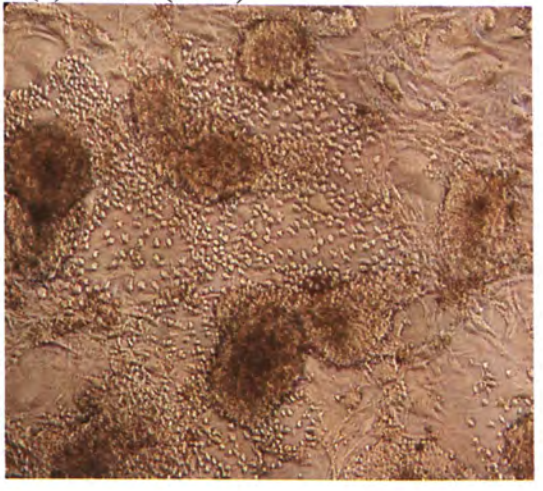
(b) with L (40x)



(c) with C17.2 (100x)



(d) with L (100x)



4.2.2.3 Non-contact co-culture

Non-contact co-culture allows free diffusion of bio-metabolites and soluble factors among cells of interest separately grown in two compartments: cell inserts of finely pored permeable membrane on wells of culture plates (Figure 17). The SDIA of C17.2, L-Wnt-3A and L cells were evaluated by seeding ES cells D3 and E14TG2a onto poly-L-ornithine- and laminin-coated wells and co-culturing with confluent growth of mitotically inactivated stromal cells pre-established on cell inserts for eight days. Cross-talks of cell foci with protruding processes were evident in co-cultures of ES cells D3 and neural precursor cells C17.2, whereas colonies of undefined contours in a flat sea of small round cells were also present (Figure 18). *In-situ* immuno-staining demonstrated the positivity of the NSC marker, nestin, in more than 60% of cell foci in all co-cultures (Figure 19).

Figure 20 showed the percentages and numbers of nestin$^+$ cell foci derived from co-cultures of ES cells D3 and the three stromal supports in four separate experiments. All SDIA-induced cultures yielded a significantly greater number of nestin$^+$ colonies as compared to 54.9% in control cultures without SDIA (C17.2 cells: 74.7%, $p = 0.029$; L cells: 64.9%, $p = 0.029$; L-Wnt-3A: 64.7%, $p = 0.029$). Among the three stromas, C17.2 displayed the strongest inducing activity on the generation of nestin$^+$ colonies, which was significantly superior to the two counterparts ($p = 0.029$). Conversely, the numbers of nestin$^+$ colonies derived from co-cultures with L cells and L-Wnt-3A cells were

comparable (p = 1.000), suggesting that Wnt-3A protein may play a little role in ES cell derivation into neural cell lineage.

The SDIA of C17.2 cells, L cells and L-Wnt-3A cells were attested on the other cell line E14TG2a in four separate experiments (Figure 21). Similar readouts were obtained. The proportions of nestin$^+$ colonies derived from the three stromas were 74.8%, 70.1%, and 68.6%, respectively, being significantly greater than 57.2% in control cultures without SDIA (C17.2 cells: p=0.029; L cells: p = 0.029; L-Wnt-3A cells: p = 0.029). Despite SDIA derived from C17.2 cells was noted to yield the highest number of nestin$^+$ colonies in the induction of ES cells E14TG2a, the extent did not reach a statistical significance as compared to those of L cells and , L-Wnt-3A cells (C17.2 vs. L cells: p = 0.343; C17.2 vs. L-Wnt-3A: p = 0.343). The comparable numbers of nestin$^+$ cell foci derived from co-cultures of ES cells E14TG2a and L cells/L-Wnt-3A cells supported the notion that Wnt-3A protein may not be crucial in the induction of ES cells into neural cell lineage.

Cell products derived from SDIA-induced cultures of ES cells D3 and E14TG2a on C17.2 cells were molecularly characterized for lineage commitment by using RT-PCR (Figure 22). They were noted to express neural cell lineage-related genes: *Pax6* and *Otx1* (neuro-ectodermal), *Nurr1* (neuronal) and *GFAP* (astroglial). CXCR4 which is the ligand of the chemokine SDF-1/*CXCL-12* was also detected in the cell product indicating cues on the migration of NSC in the CNS. *Brachyury* and *α-fetoprotein* which are genetic markers of mesodermal and endodermal cells, respectively, were not

amplified in the cell products suggesting relatively pure populations of neural cell lineage being free of any mesodermal and endodermal cells. However, weak expression of *Oct-4* was noted implying residual ES cells in the cell products.

Figure 17 Non-contact co-culture system. This is basically consisted of cell inserts (arrow) with permeable membrane hung over the tissue culture plate. The whole apparatus allows freely diffusible biometabolites across the membrane but keeps two cell populations of interest separated.

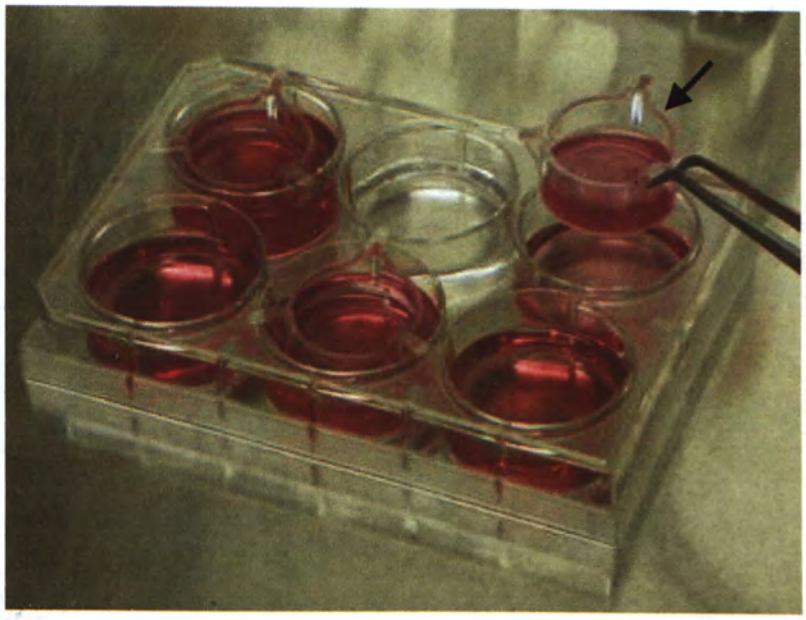
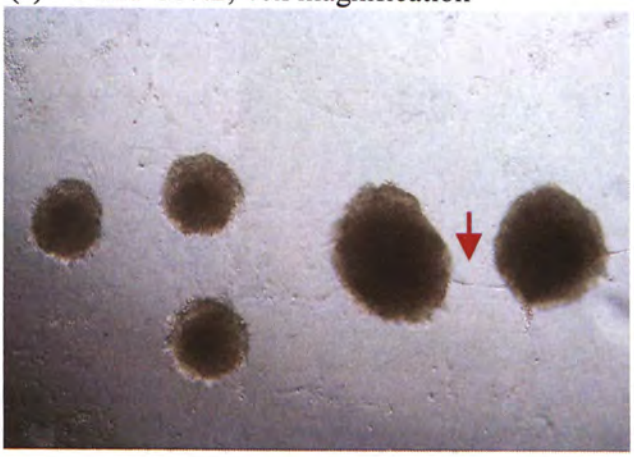
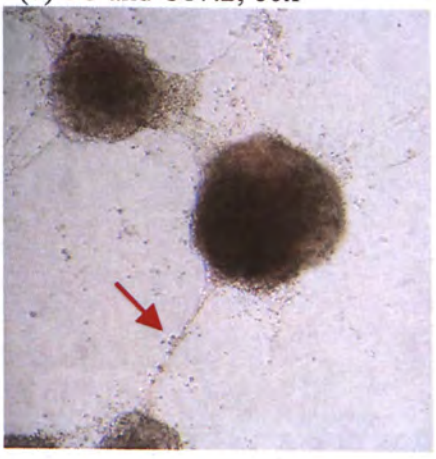


Figure 18 Cell foci derived from non-contact co-cultures of D3 for eight days. In C17.2 stroma, some cells formed network with neighbouring foci, as red arrows in (a) and (b). In L (c) or L-Wnt-3A (d), most colonies had undefined contours and small round cells were observed in the background.

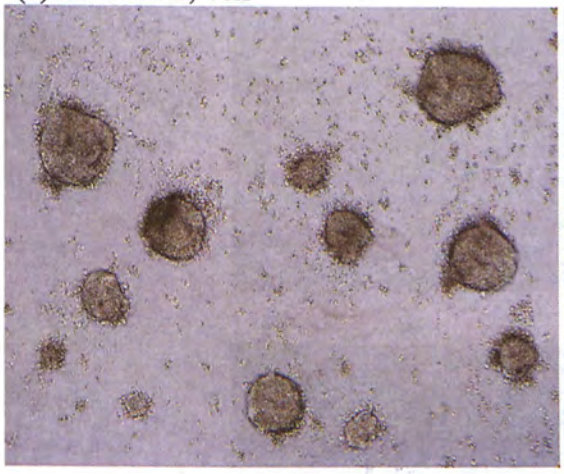
(a) D3 and C17.2; 40x magnification



(b) D3 and C17.2; 60x



(c) D3 with L; 40x



(d) D3 with L-Wnt-3A; 40x

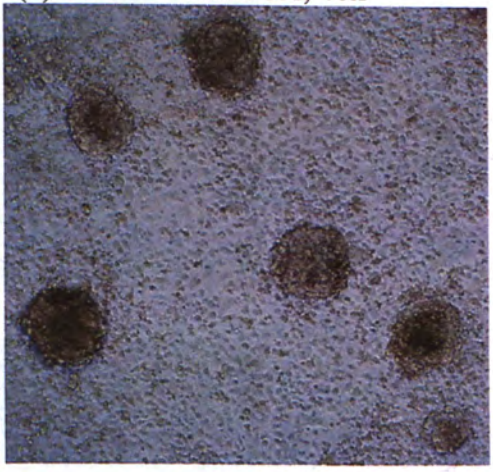
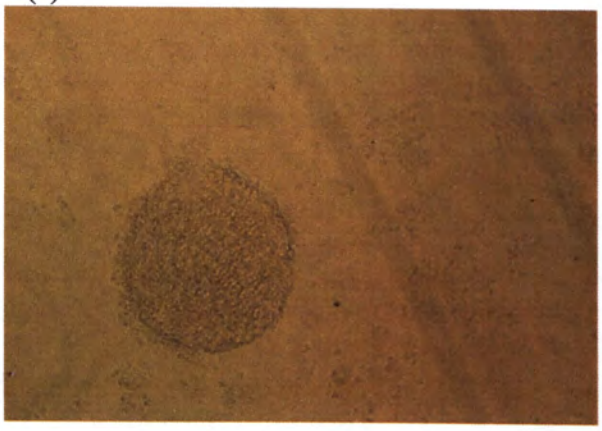
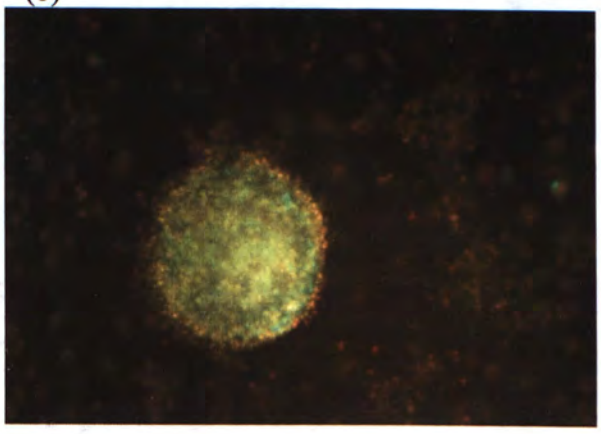


Figure 19 *In-situ* nestin staining of cell colonies derived from non-contact co-cultures of ES cells D3 and C17.2. (a) phase contrast image of cell focus, (b) nestin^{+} focus (green fluorescent, counter-stained with PI) and (c) strongly nestin^{+} focus with remarkable sprouting of processes at 40x magnification.

(a)



(b)



(c)

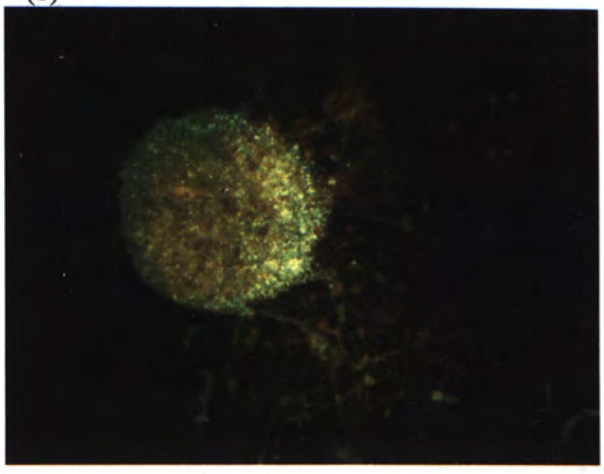
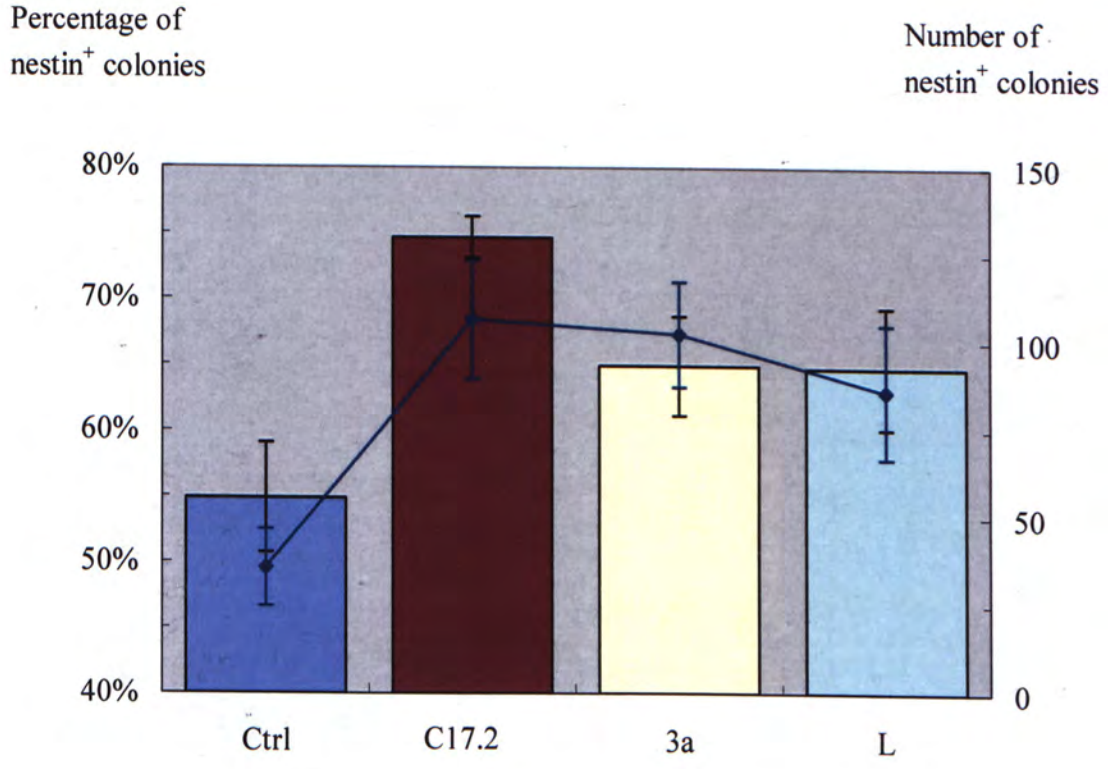


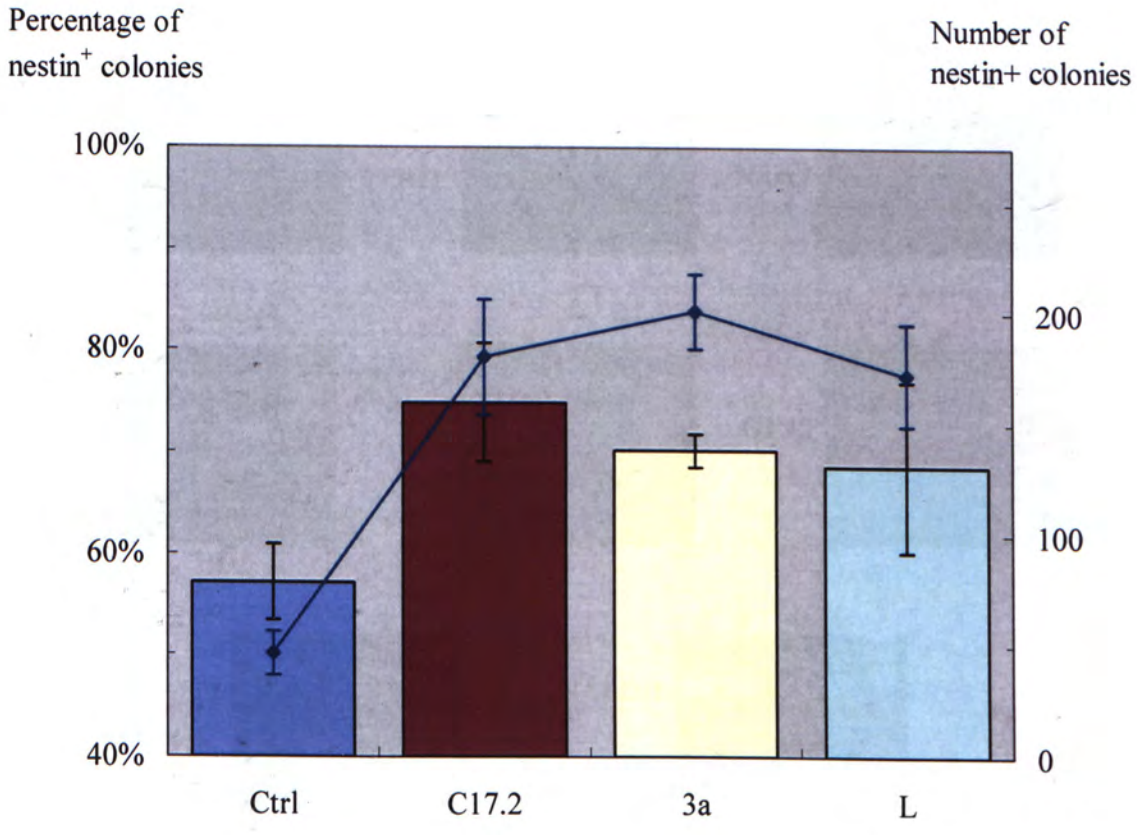
Figure 20 Proportion and number of nestin$^+$ cell foci derived from four separate experiments in non-contact co-cultures of ES cells D3 and stromal cells, C17.2, L, and L-Wnt-3A.



Colored bar: nestin$^+$ cell foci in percentage

Blue line: number of positive cell foci

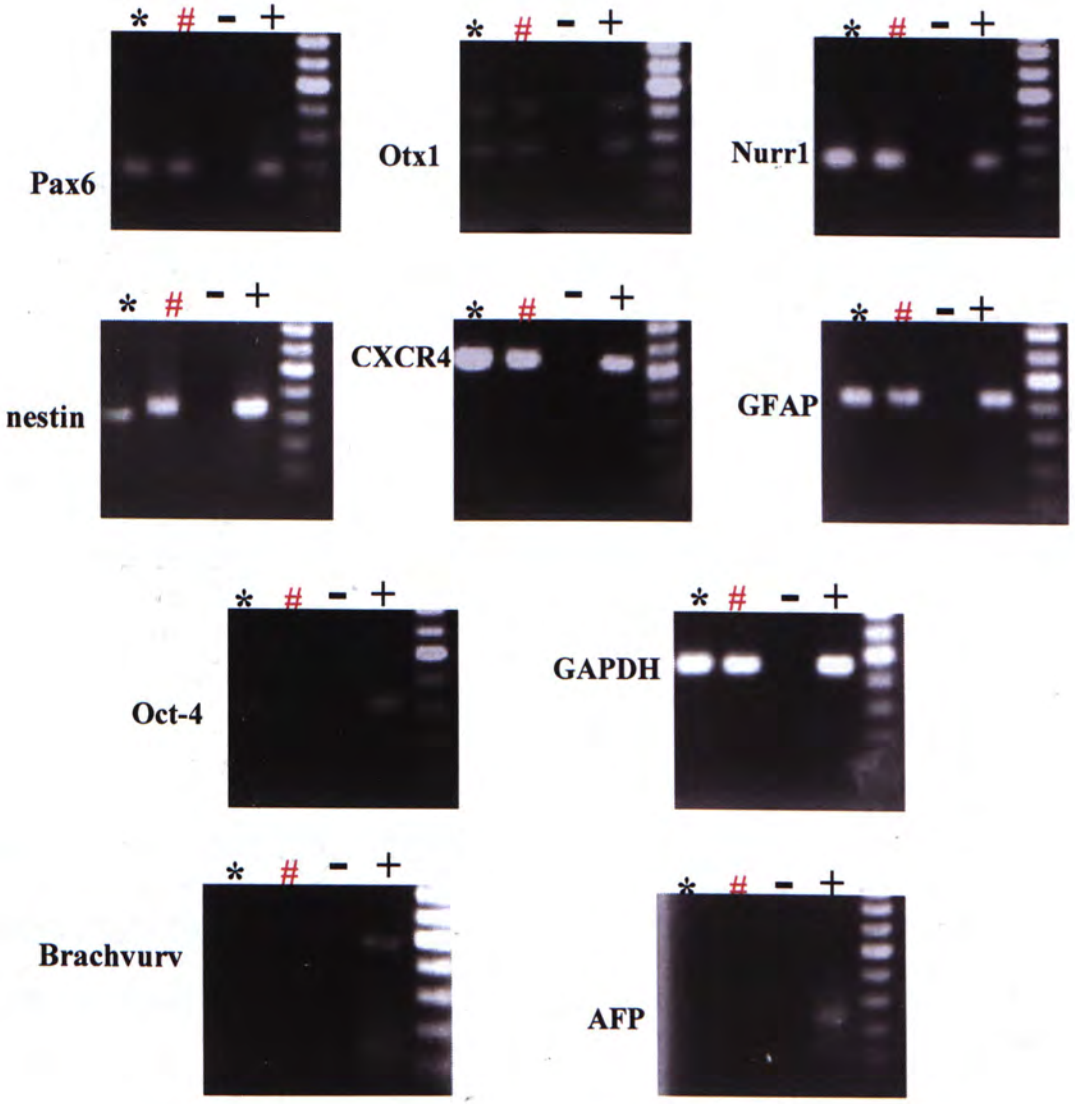
Figure 21 Proportion and number of nestin^{+} cell foci derived from four separate experiments in non-contact co-cultures of ES cells E14TG2a and stromal cells, C17.2, L, and L-Wnt-3A.



Colored bar: nestin^{+} cell foci in percentage

Blue line: number of positive cell foci

Figure 22 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products of SDIA-induced cell products. (Pax6, Otx1: neuroectodermal markers; Nurr1: neuronal marker; GFAP: astroglial marker; nestin: neural stem cell marker, CXCR4, a chemokine receptor; Oct-4: ES cells marker; Brachyury: mesodermal marker; AFP: endodermal marker; GAPDH: a house keeping gene)



*: D3 ES cell line

#: E14TG2a ES cell line

4.2.2.4 Cultures in CM

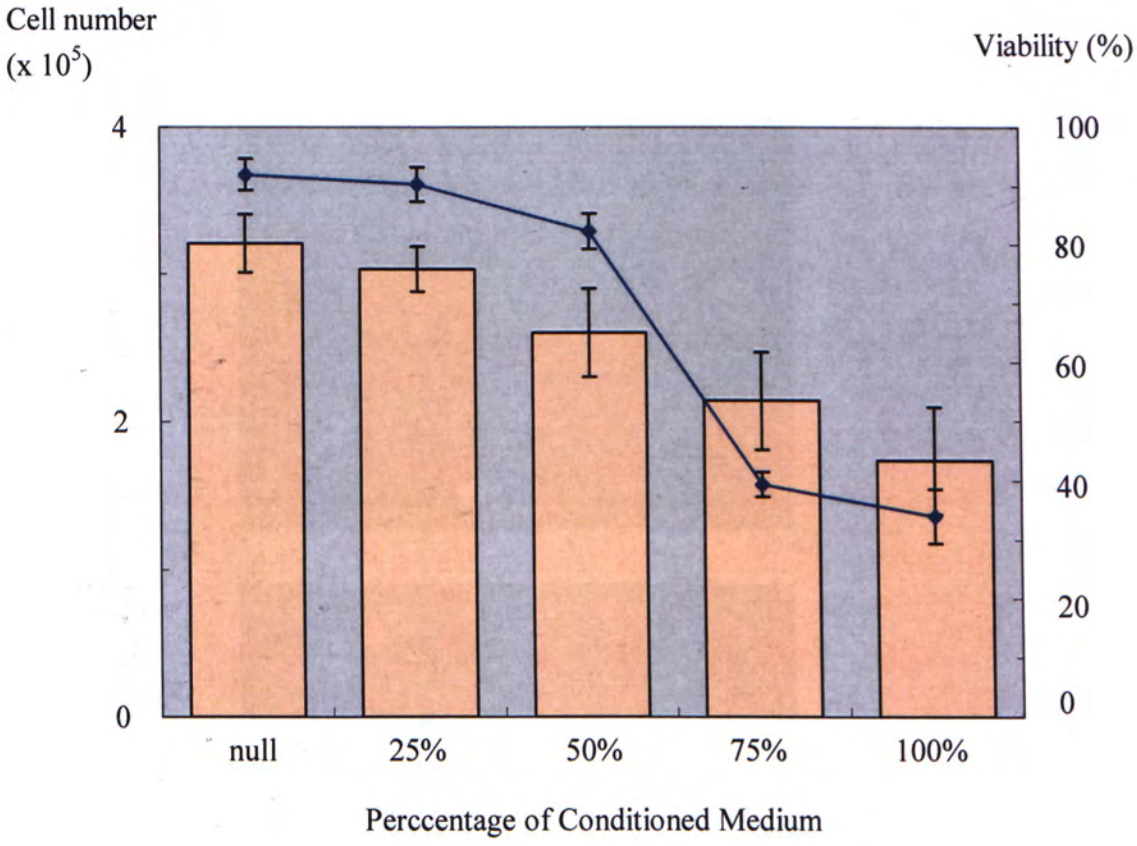
Spent media of four-day cultures of confluent growth of irradiated C17.2 cells, L cells and L-Wnt-3A cells were collected on every two alternate days. Collections from six batches were pooled and used as the CM. The viability of 1 x 10^5 ES cells D3 cultured in wells of serially diluted C17.2-derived CM for four days showed that half-diluted CM could best support cell growth pertaining to the likely endogenous growth-promoting factors and inhibitory bio-metabolites or toxins being diluted to an optimal titrate (Figure 23). Besides, neural induction was also evident as shown in Figure 24.

In the comparative study of the efficacy of three CM, readouts in a similar pattern were noted in the induction of ES cells D3 and E14TG2a in three separate experiments (Figures 25 and 26). The mean proportions of nestin$^+$ cell foci of ES cells D3 cultured in CM derived from C17.2 cells, L cells and L-Wnt-3A cells were 69.7%, 61.8% and 61.6 %, respectively, being greater than 55.2% in the controls using no CM, but not statistically significant (C17.2 cell-derived CM: $p = 0.1$; L cell-derived CM: $p=0.2$; L-Wnt-3A cell-derived CM: $p=0.2$). So did the percentages of nestin$^+$ colonies derived from ES cell E14TG2a cultures in three CM as compared to 54% in the control cultures without CM (C17.2-derived CM: 72.6%, $p=0.1$; L cell-derived CM: 63.7%, $p = 0.2$; L-Wnt-3A cell-derived CM: 69.2%, $p = 0.1$).

The incidences of nestin$^+$ cell foci of ES cells, D3 and E14TG2a, cultured in L cell- and L-Wnt-3A cell-derived CM were comparable (D3: $p = 1.000$; E14TG2a:

p=0.400). However, the number of nestin^{+} cell foci derived from ES cells D3 and E14TG2a were significantly greater in CM of L-Wnt-3A cells than CM of L cells (L-Wnt-3A cell-derived CM vs. L cell-derived CM: D3: 117 ± 10.5 vs. 80 ± 2.1; p = 0.013; E14TG2a: 60 ± 4.4 vs. 30 ± 4.7; p = 0.008), suggesting the growth-promoting role of Wnt-3A.

Figure 23 Mean cell viability and number of 1 x 10^5 ES cells D3 cultured for four days in wells of the C17.2-derived CM at various concentrations in three separate experiments.



Colored bar: Total cell number

Blue line: Viability

Figure 24 Morphology and *in-situ* immunostaining for nestin of cell foci from cultures of ES cells D3 in C17.2-derived CM for eight days. (a) phase contrast image, (b) a nestin^{+} colony (green fluorescent, counter-stained with PI) with extensive protruding processes, and (c) a brightly positive colony with radial processes. Arrows are pointing to the radial processes, some were only visible after immunostaining.

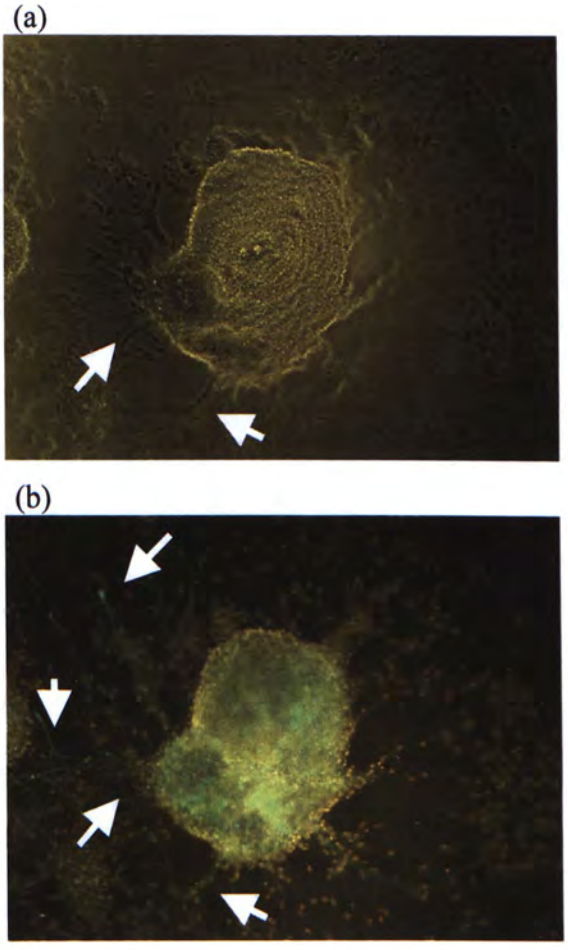
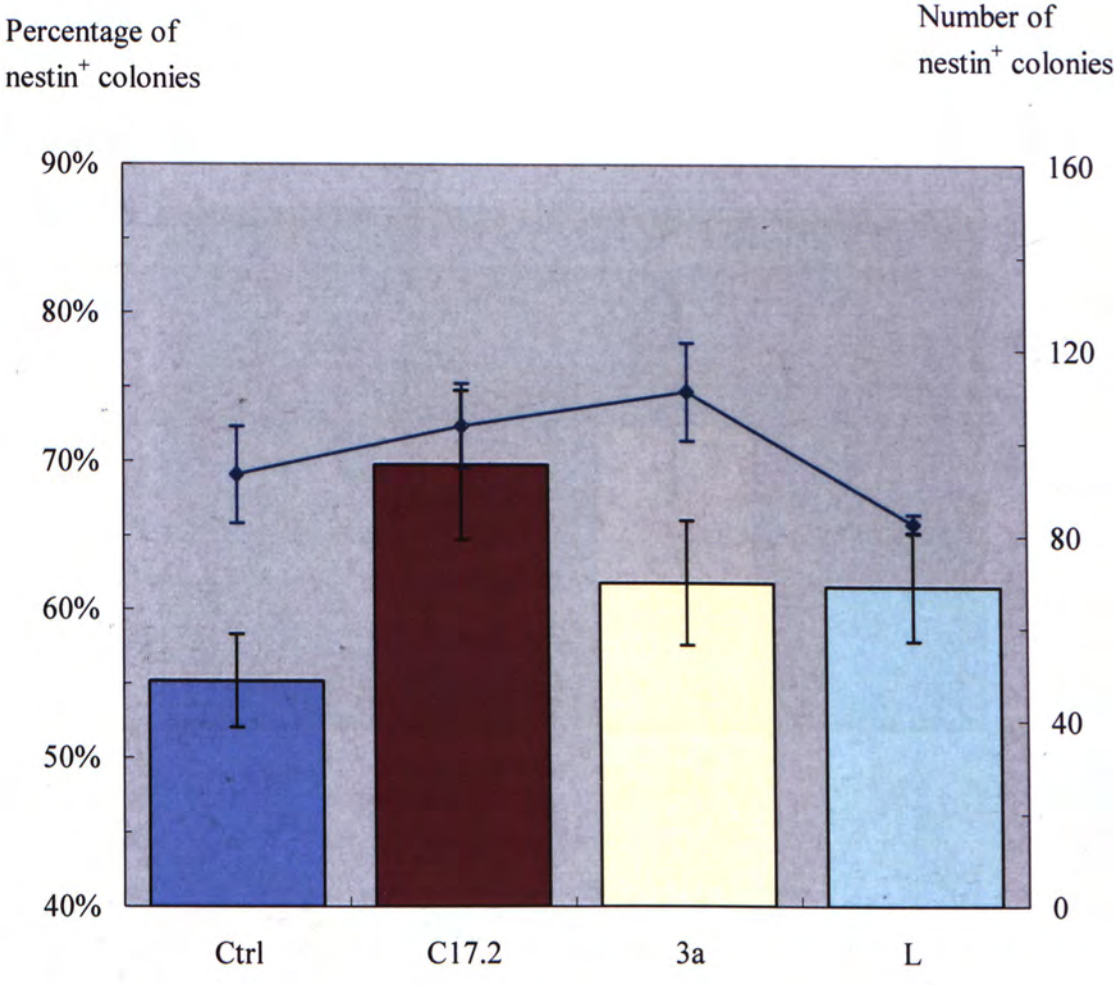


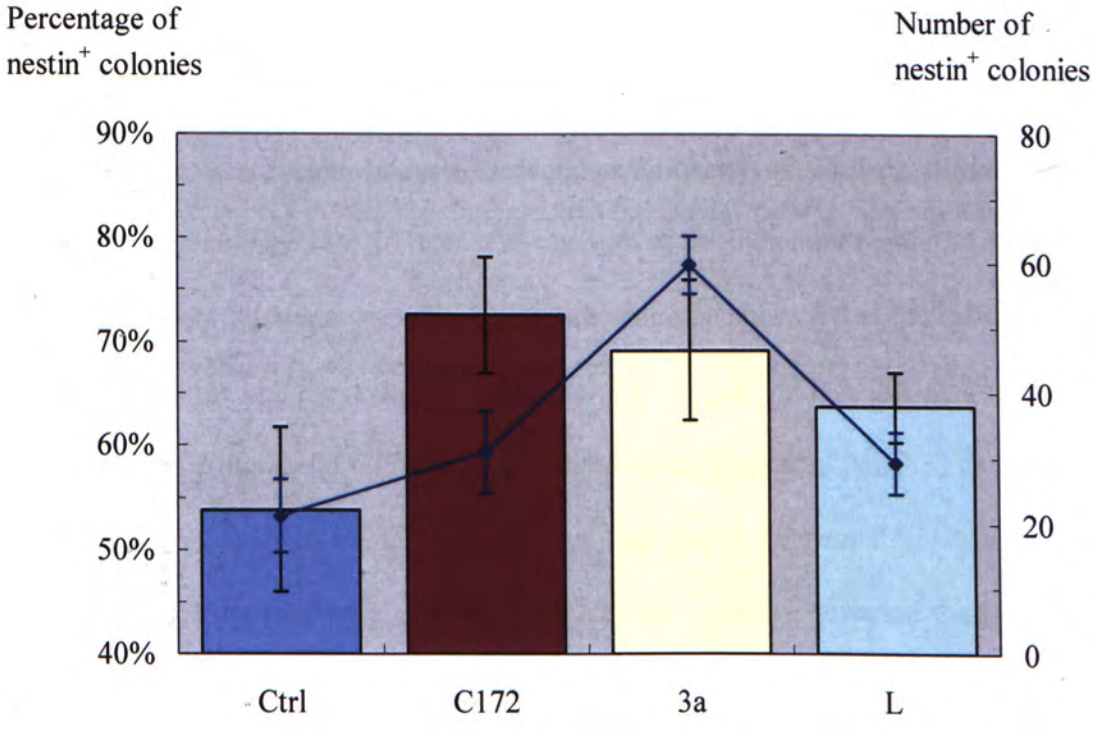
Figure 25 Proportion and number of nestin$^+$ cell foci derived from cultures of ES cells D3 in conditioned media prepared from C17.2 cells, L cells and L-Wnt-3A cells in three separate experiments.



Colored bar: nestin$^+$ cell foci in percentage

Blue line: number of positive cell foci

Figure 26 Proportion and number of nestin$^+$ cell foci derived from cultures of ES cells E14TG2a in conditioned media prepared from C17.2 cells, L cells and L-Wnt-3A cells in three separate experiments.



Colored bar: nestin$^+$ cell foci in percentage

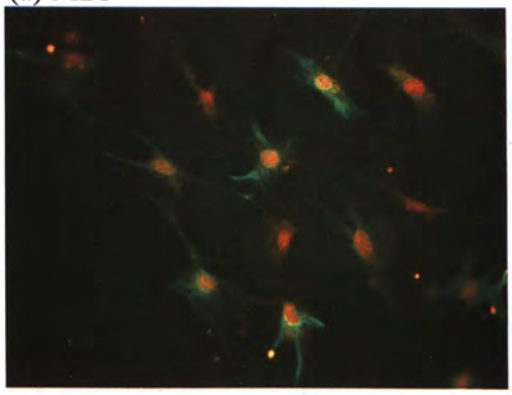
Blue line: number of positive cell foci

4.3. ES cell Differentiation

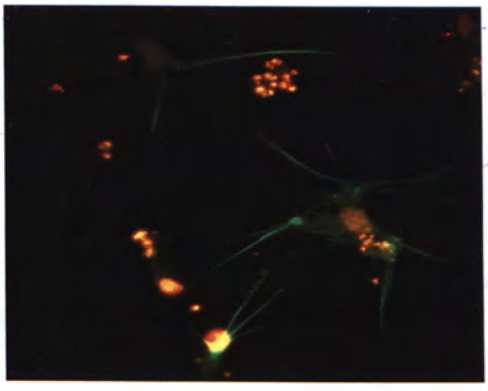
C17.2-mediated SDIA-induced cell products of ES cells D3 were enzymatically segregated using trypsin and 2 x 10^5 discrete cells in 2 mL serum-free N2B27 medium, without any supplement of growth factors or cytokines, were seeded in triplicate onto poly-L-ornithine- and laminin-coated coverslips in wells of 24-well culture plates. Cultures were maintained for 10 days with changes of the induction medium III (N2B27) medium for every alternate day. *In-situ* immuno-staining showed that the differentiated cell products expressed the neuronal markers of TuJ-III and MAP-2, DA neuron marker of TH, astrocytic marker of GFAP and oligodendrocytic marker of MBP. The immuno-positive cells existed as single cells, clusters and sheets (Figure 27). Microscopic examination of five randomly selected fields of 10x objective revealed that the mean percentages ± one standard deviation of tubulin$^+$ cells, MAP-2$^+$ cells, TH$^+$ cells, GFAP$^+$ cells and MBP$^+$ cells were 28% ± 10%, 20% ± 10%, 12% ± 4%, 5% ± 4% and 12% ± 7%, respectively, suggesting that ES cells could be induced and differentiated into neural cell lineage through the SDIA of the neural precursor cell line C17.2.

Figure 27 *In-situ* immuno-staining of SDIA-induced cell product of ES cells D3. (a) MBP-expressing oligodendrocytes, (b) GFAP-positive astrocytes, (c) immature TuJ-III$^+$ and (d) mature MAP2$^+$ neurons, and probably (e) TH$^+$ dopaminergic neurons, were observed in N2B27 medium for further 10 days. All markers are green fluorescent and slides were counter-stained with PI to show the nuclear structure.

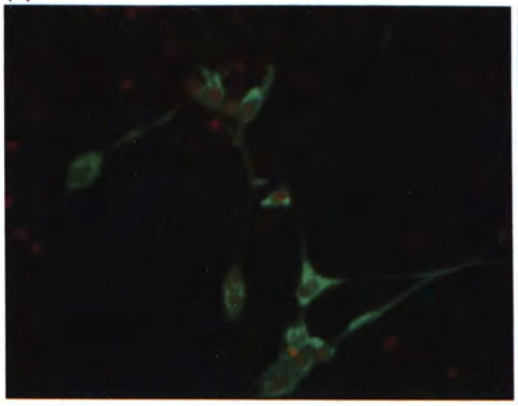
(a) MBP



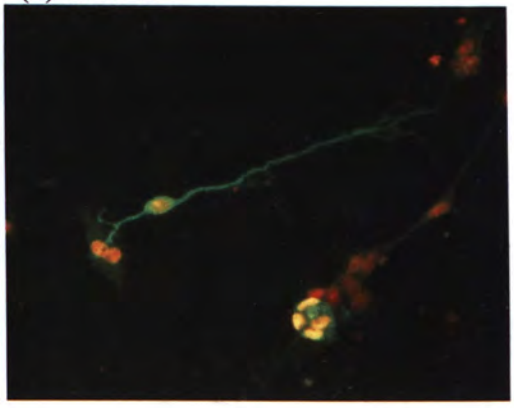
(b) GFAP



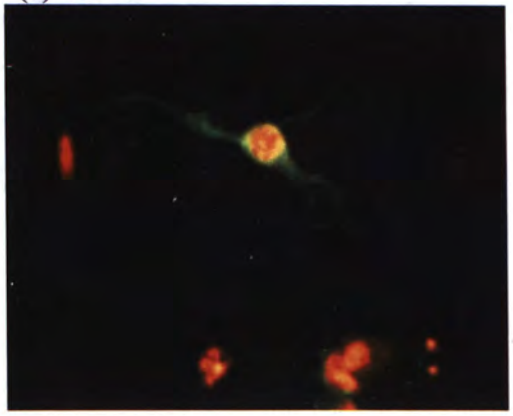
(c) TuJ III



(d) MAP-2



(e) TH



4.4 *In vivo* study of ES cell-derived cell products

4.4.1 Animal preparation

Twenty-four adult male ICR mice of mean age 12 weeks were divided into three arms at random namely, normal control group, ischaemic group and transplanted group. Global cerebral ischaemia was induced to 18 mice a week prior to cell implantation (Figure 28). The operated animals exhibited signs of disturbance of consciousness including drowsiness, paucity of movement and even coma which was evident in a mouse. Having undergone transient BCCAO, 12 mice survived accounting to the survival rate of 66.7%.

4.4.2 Cell preparation

One day in advance of the transplantation, SDIA-induced and differentiated cell products of ES cells D3 co-cultured with C17.2 were labeled with BrdU. Trypan blue dye exclusion test and immuno-staining of the BrdU-labelled cell products illustrated 74% viable cells and 75% nestin^{+} cell foci, respectively.

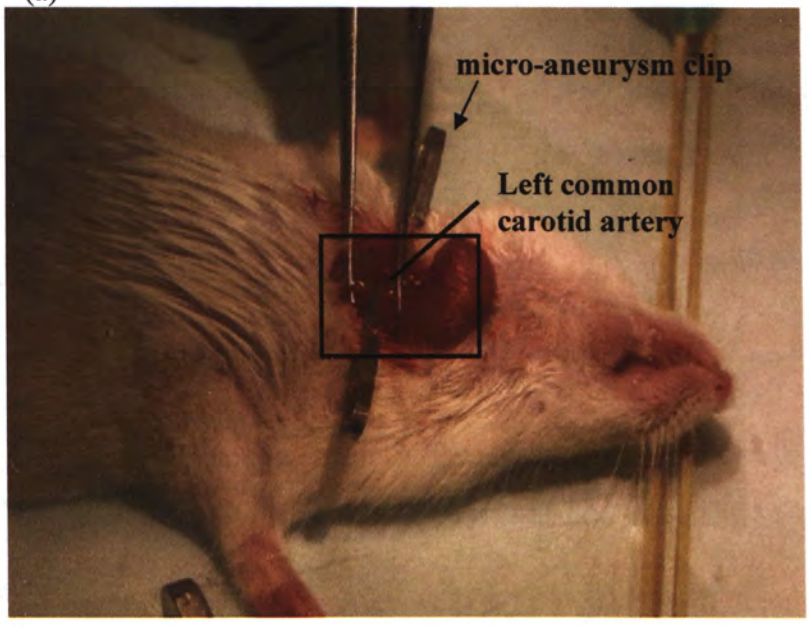
4.4.3 Cell implantation

Having been mechanically dislodged from the culture wares, five µL of 5 x 10^5 viable SDIA-induced and differentiated cell products of ES cells D3 were implanted

onto the mouse striatum, at the pre-determined stereotaxic co-ordinates, of transplanted group (Figure 29).

Figure 28 Global cerebral ischaemia induced by transient BCCAO for 15 minutes. Picture (a) showing that two micro-aneurysm clips were placed over the bilateral common carotid arteries in the neck. (b) A magnified view of the occlusion region illustrating that blood was blocked from flowing from the lower part of the body to the brain.

(a)



(b)

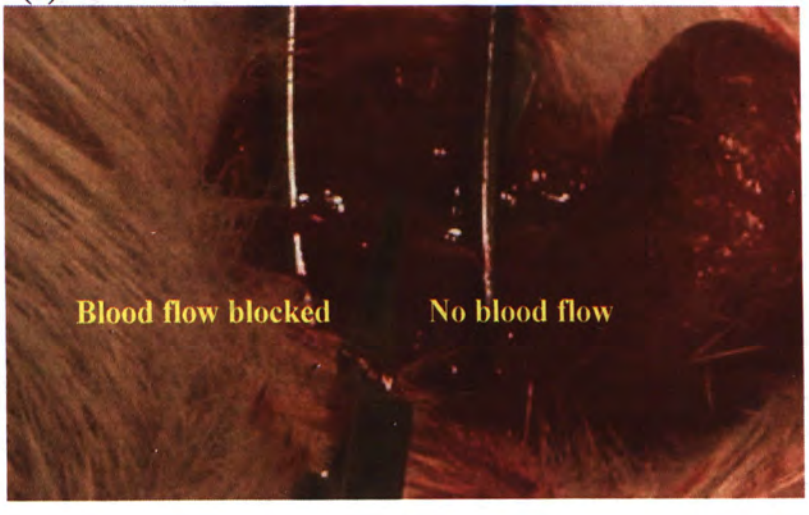
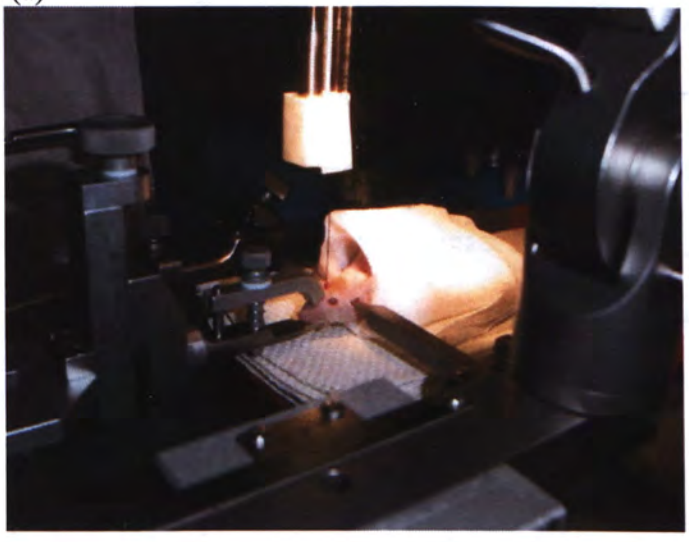
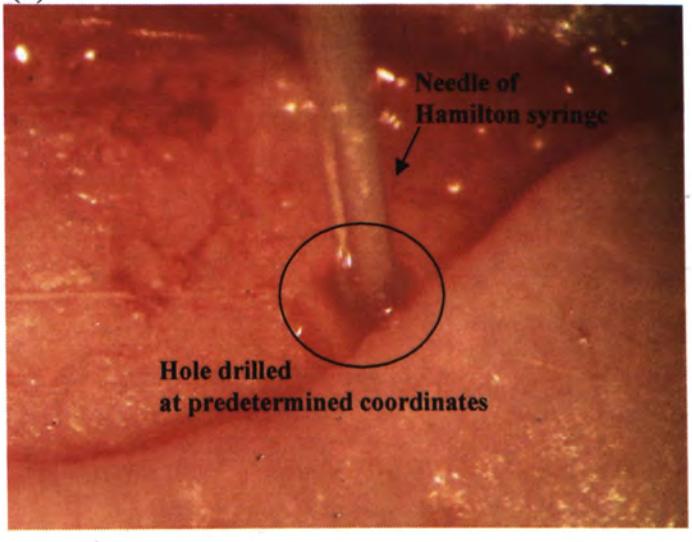


Figure 29 Neuro-surgical procedure of cell transplantation. (a) An ischaemic mouse was sedated and immobilized onto the stereotaxic apparatus. (b) ES cell D3-derived cell product was implanted intracranially onto the straitum by a Hamilton syringe through a hole drilled at the pre-determined co-ordinates.

(a)



(b)



4.4.4 Behaviour Monitoring

Two weeks post-implantation, the spatial learning and memory ability of mice of interest were assessed using the water maze system (Figure 30). Upon completion of daily training on three occasions, the number of episodes for mice entering cue de sacs and the time taken to find the way out during the manoeuvre on the following two days were reckoned. Figure 31 shows that the incidences of error committed by mice among the three studied arms on day four and day five were comparable. Figure 32 illustrates the time taken by ischaemic mice to go through the maze were relatively stable over five days. On the contrary, there was a progressive reduction of travelling time spent by both normal mice and ischaemic mice having undergone ES cell-derived cell therapy. Basically, both normal control mice and cell therapy-treated ischaemic mice could find their way out in less than a minute on day five, which were significantly shorter than that taken by the ischaemic counterparts (mean value in second: cell therapy-treated ischaemic group vs. ischaemic group: 43 vs. 161, $p=0.008$, $n=12$; normal control group vs. ischaemic group: 56 vs. 161, $p=0.009$, $n=12$).

Figure 30 The water maze system for testing the spatial learning and memory ability of mice. (a) The overall view of the system, (b) a test mouse swam shortly away from the start point in the far left of the maze, (c) a test mouse negotiated its way out of a cue de sac, (d) a test mouse eventually made it and climbed up the ladder at the finishing spot.

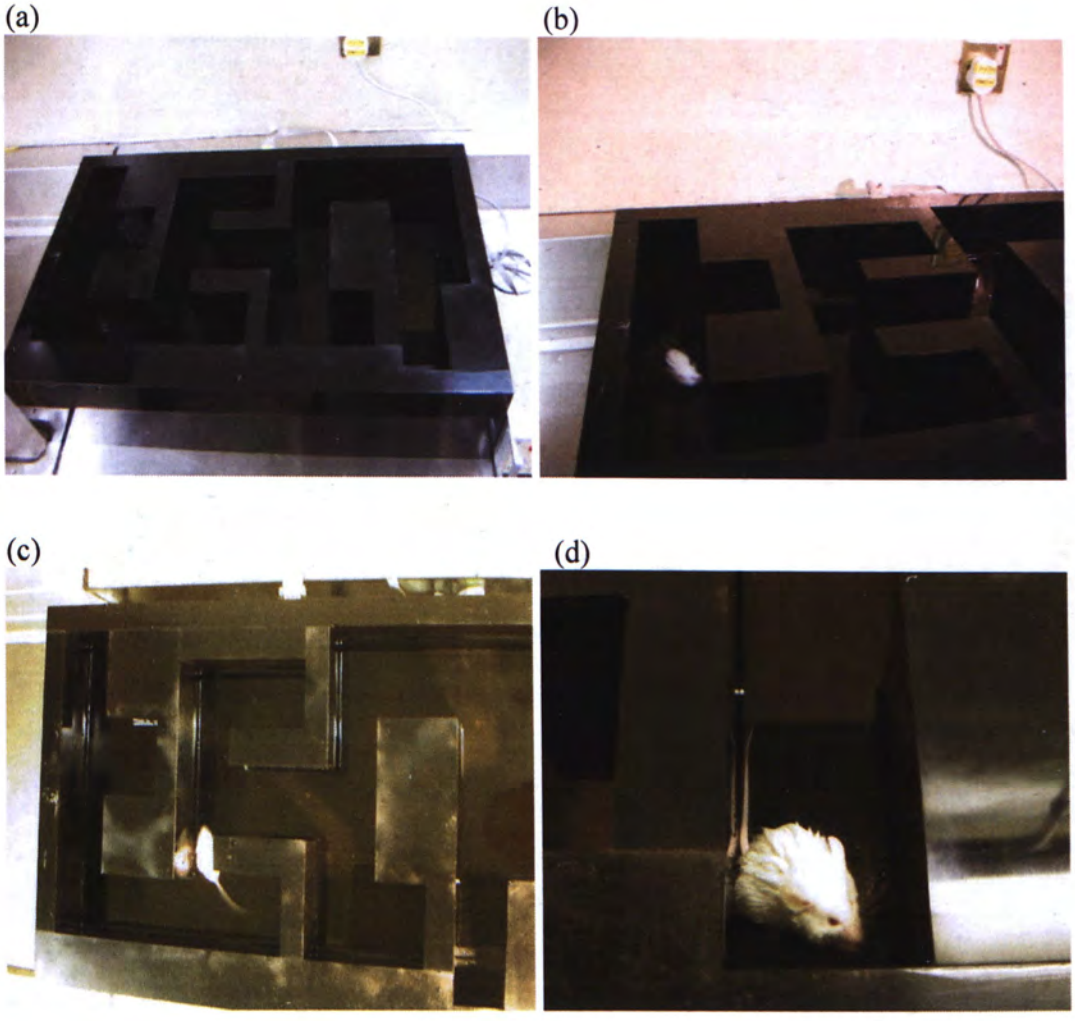


Figure 31 The average number of errors, in terms of entering cue de sacs of the water maze system, committed by the tested mice (six each in three studied arms on five consecutive days). Control group are normal mice. Ischaemic (IS) group and transplanted ischaemic (IS+C) group are mice injected intracranially with normal saline and ES cell-derived cell product, respectively, two weeks post-induction of global cerebral ischaemia.

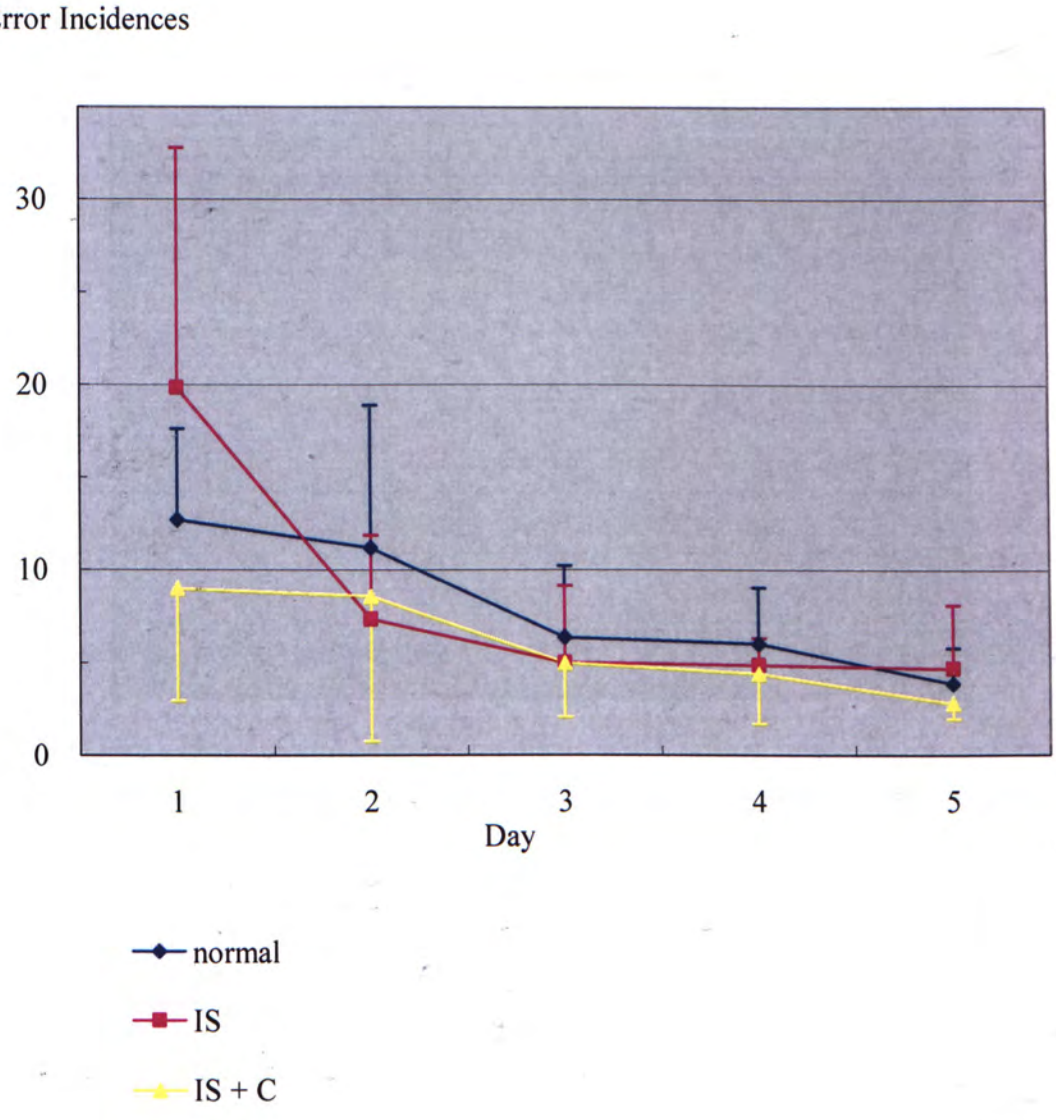
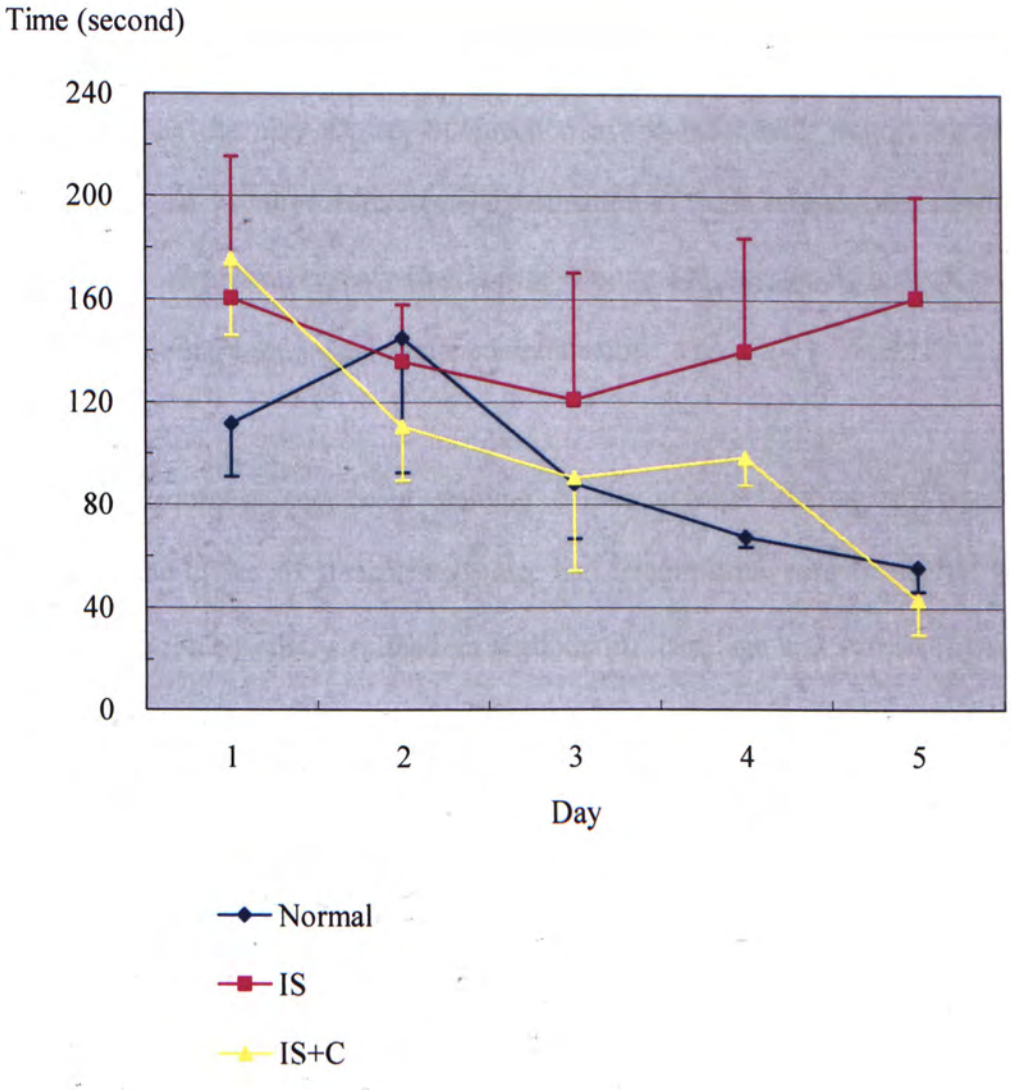


Figure 32 The average time taken to find the way out and climb up the ladder at the finishing spot by the tested mice (six each in three studied arms on five consecutive days). Control group are normal mice. Ischaemic (IS) group and transplanted ischaemic (IS+C) group are mice injected intracranially with normal saline and ES cell-derived cell product, respectively, two weeks post-induction of global cerebral ischaemia.



4.4.5 Histology of cell-implanted brain

Shortly after behavioral assessment mice were euthanized and *trans*-cardiacally perfused to strip off peripheral blood. Heads were decapitated and the whole brains were removed from the skulls for histological evidence of implantation of ES cell-derived cell product.

Figure 33 shows that BrdU-labelled ES-derived cell products predominantly localized along the needle track of injection in the brain with minor degree of cell migration to the vicinity. Meticulous examination of brain tissues contralateral to the site of injection revealed scanty $BrdU^+$ cells (Figure 34), suggesting a small number of cells are migrating from injection site contralaterally.

Haematoxylin and eosin staining of the coronal sections of brain tissues illustrated one of the six transplanted mice had teratomatous component and produced teratomas, included ciliary epithelium (endoderm), cartilage and stromas (mesoderm), and squamous epithelium (ectoderm) (Figure 35).

Figure 33 Immuno-staining of coronal sections of brain tissues three weeks post cell implantation using FITC-conjugated anti-BrdU labeling (green fluorescent). $BrdU^+$ cells are mainly localized in the injection site, and some showed limited migration (arrows in (d)).

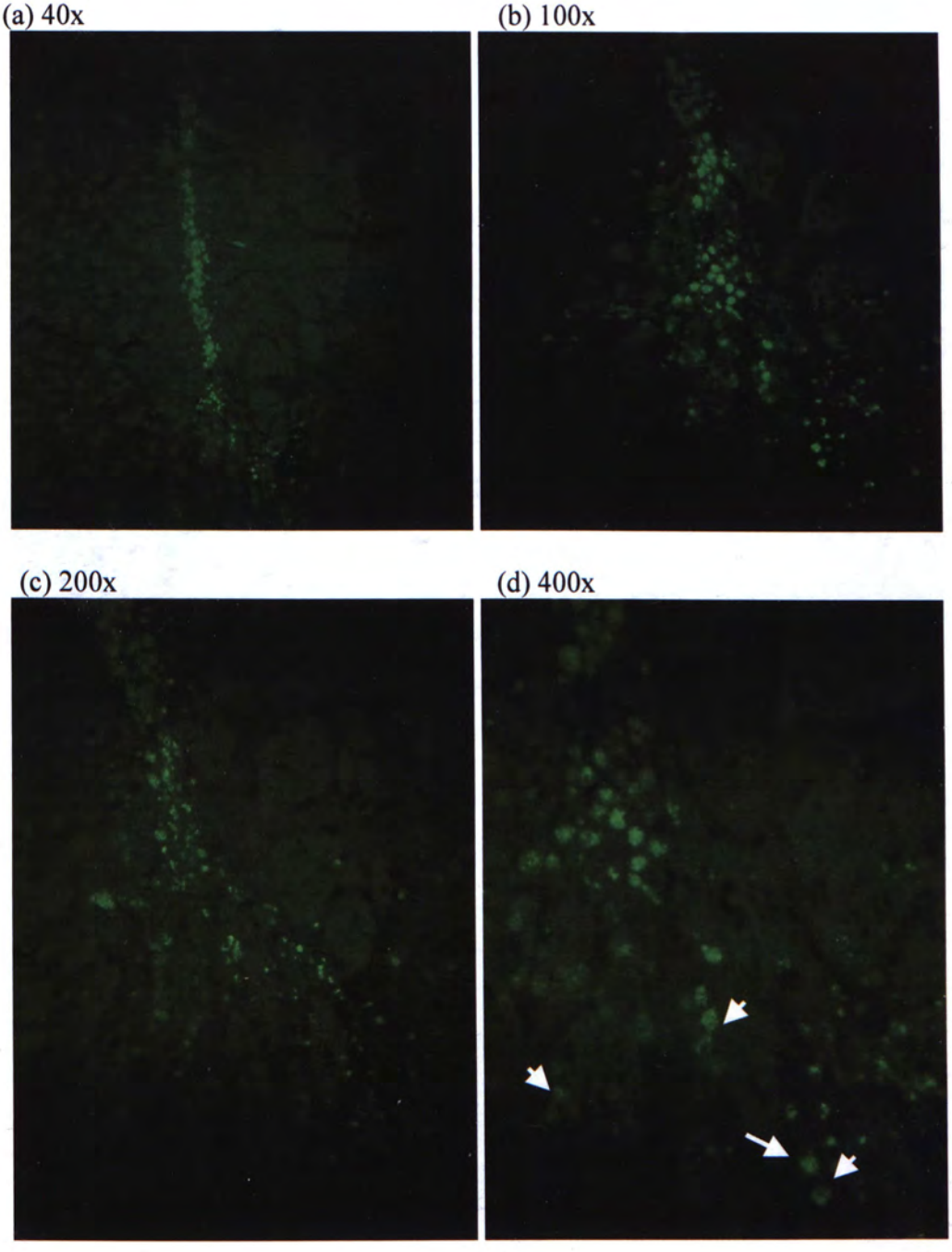
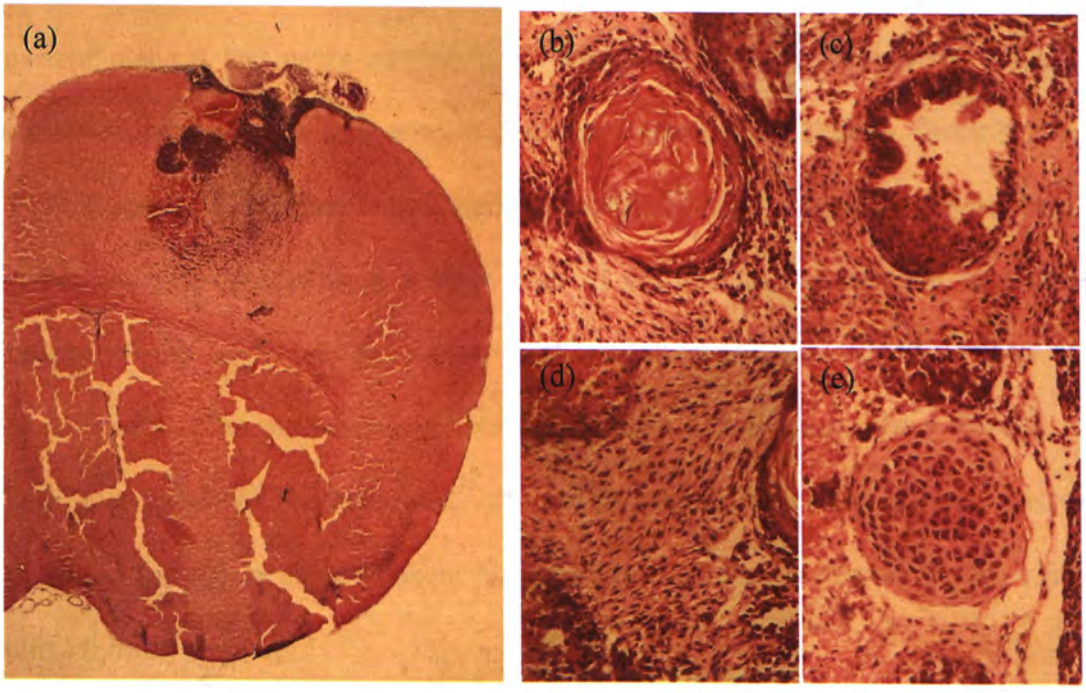


Figure 34 Immuno-staining of coronal sections of brain tissues three weeks post cell implantation using FITC-conjugated anti-BrdU labelling (green fluorescent). Results provide evidence on some degree of migration of a small quantity of grafted cells contralaterally, as shown by the white arrows.

(a) 40x magnification

(b) 100x magnification

Figure 35 Haematoxylin and eosin staining of coronal sections of the mouse brain tissue three weeks after intracranial implantation of ES cell D3-derived cell product. Teratomas in one of the transplanted mice (a) showing neural rosettes, (b) squamous, (c) ciliary epithelium, (d) stromas, (e) cartilage.



Chapter 5 Discussion

The discovery of stem cells has triggered high expectations, from both the scientific and medical arenas to the general public at large, as a novel treatment modality for neural diseases and disorders pertaining to either cell loss or degeneration, which current medical and neuro-surgical interventions deliver little therapeutic efficacy to survivors harassed with long-term to permanent neurological deficits (Svendsen et al. 1999; Gepstein 2002; Haas et al. 2005). Basically, ES cells, which are derived from an embryonic cell population at a stage prior to any lineage commitment to particular tissues of the body, are by nature more primitive and versatile than their adult counterparts. The availability of rodent-, primate- and human-derived ES cell lines helps manipulate ES cell cultures *in-vitro* and produce high yields of differentiated cells of neural lineage in large numbers for pre-clinical studies in disease models prior to clinical trials.

There is a plethora of reports in the literature describing the maintenance, induction and differentiation of ES cells using different methods and procedures employing chemical stimulants, growth factors, signalling molecules and regulators, inducing factors and/or genetic manipulation. Apparently, all these manoeuvres were executed to simulate the *in-vivo* developmental microenvironments of ES cells and lineage-restricted precursors and end-stage mature cells of interest. The efficacy varies in terms of yield, purity, viability and functionality, not to mention the ease of performance and processing time required.

ES cell maintenance is a very important process to propagate them *in-vitro* in the way that the stemness of the progenies is identical to that of their predecessors without any differentiation. It secures the provision of quality yield of undifferentiated ES cells for down-stream experiments. However, ES cells are very vulnerable to the *in-vitro* conditions of propagation. A slight variation may result to undue differentiation (Pollard 1997; Marshak 2001).

ES cells are normally propagated by co-culturing with mitotically inactivated PMEF or mouse embryonic fibroblast cell lines in serum-based medium supplemented with LIF. In mouse ES cells, LIF plays a crucial role in preventing differentiation by activating the LIF-STAT3 signalling pathway involved in the *in-vitro* self-renewal process (Williams et al. 1988; Gough et al. 1989; Yoshida et al. 1994; O'Shea 2004; Cartwright et al. 2005). That LIF was detected in cultures of PMEF and the mouse embryonic fibroblast cell line STO was reported (Smith et al. 1987; Smith et al. 1988). It is suggestive that LIF and other uncharacterized anti-differentiating factors derived from PMEF and STO may help prevent ES cell differentiation (Lim et al. 2002). Data of this study showed that STO was less efficient than PMEF in preventing ES cell differentiation as shown by the comparatively higher numbers of SSEA-1$^-$ cells in the maintenance cultures of ES cells on STO support. It has been reported that anti-differentiating effect of feeders could progressively diminish and be lost upon continuously multiple passages (Williams et al. 1988; Joyner 1999). The observation in this study provides a note of caution in assuming the stemness of ES cells maintained on

STO of an unknown passage number.

The nature of serum in the culture medium plays a pivotal role of ES cell maintenance. There are many uncharacterized bio-metabolites, factors, substances and molecules, either growth-promoting or growth-inhibiting, which vary significantly from lots, batches and manufactures (Ward et al. 2002). It was evident in this study that ES cell maintenance cultures supplemented with un-tested serum displayed a slower propagation rate and even signs of differentiation. Optimally screened serum is mandatory to maintain ES cells and avoid differentiation.

Propagation of ES cells in serum-free condition was advocated (Ward et al. 2002; Cheng et al. 2004). In this study, an alternative approach was used to propagate ES cells in suspension cultures by employing the chemically reconstituted serum-replacer (SR) in lieu of serum and feeder support on low adherent culture wares to prevent spontaneous differentiation mediated by cell adhesion. The extents of proliferation and stemness of first few passages of ES cells propagated in serum-free condition were comparable to those derived from serum-based cultures on feeder support. However, SSEA-1 immuno-reactivity decreased progressively in subsequent ES cell passages suggesting the insufficiency of serum-replacer in maintaining ES cell integrity and the importance of feeder support to augment LIF to prevent spontaneous differentiation of ES cells. Data of this study were in contrast to that of Christopher and co-workers (Ward et al. 2002). The observation may be attributable to the density of seeded cell and the nature of culture. In their study, a relatively high density of ES cells, 3×10^5/ml was seeded in a

100mm dish. ES cell ES cell lines were weaned by subsequent passaging in media of serially increased SR percentage (FCS/SR: 75/25%, 50/50%, 25/75%, and 0/100%) before removal of feeders. Besides, the ES cells were cultured on gelatinized dish, instead of suspension culture. However, the proportion of ES cells derived from serum-free condition expressing ES cell-specific markers of Oct-4 and SSEA-1 were not studied.

Throughout the project, ES cell lines, D3 and E14TG2a, were propagated on mitotically inactivated PMEF in propagation medium supplemented tested serum and LIF. The medium was changed at least once daily.

In the CNS there are many neurons, however glia including astrocytes, oligodendrocytes, ependymal cells and microglia outnumber neurons by ten-fold and their precise roles and functions in the CNS have not yet been fully elucidated. They are considered to be sleeping giants with immense and enormous potentials. Apart from synthesizing neuro-transmitters, neurotrophins, growth factors, cytokines, adhesion molecules, the respective receptors and ligands are regulating the contents in their extra-cellular territories, glia are also noted in enveloping synaptic junctions, myelinating and insulating axons, providing linings of fluid-filled ventricles, directing cell migration and phagocytosing debris left by dead or degenerating neurons and glia (Siegel 1999). They play an indispensable role in neural tissue repair and regeneration in the CNS system, which effects may be attributable to the SDIA.

Neural induction of mouse ES cells by SDIA of the stromal cell PA6 derived from skull bone marrow was first reported by Kawasaki and co-workers in Japan (Kawasaki et al. 2000). It was noted that PA6-derived SDIA could induce efficient neuronal differentiation of co-cultured ES cells in serum-free conditions without use of either RA or EB. The effects are likely attributed to the production of neural cell-inducing factors in both labile soluble and cell surface-anchored forms or the secretion of factors secondarily tethered to the ES cell surface, which then elicit neural differentiation (Kawasaki et al. 2000; Kawasaki et al. 2002; Morizane et al. 2002).

The molecular basis of induction remains to be understood. It was reported that the mesoderm-differentiating BMP-4 suppressed SDIA-induced neuralization and promotes epidermal differentiation, suggesting that SDIA acts by stabilizing the ectodermal fate and protecting ES cells from mesodermalizing influences (Tropepe et al. 2001). However, the scenario brings forth the platform of employing stromal support mimicking the microenvironment of developing or injured brain for induction of ES cells into neural cell lineage. ES cells will first be induced to the ectodermal commitment using SDIA. They are then allowed to adopt a default differentiation to neural fate, be expanded and selected in *in-vitro* cultures.

RT-PCR was performed to characterize the neural-lineage related gene expression of the neural progenitor cell line C17.2, L cells and the L-Wnt-3A secreting biologically active Wnt-3A proteins. Gene expressions of neurotrophic and neutron-protective factors (*BDNF*, *GDNF*, *CNTF*, *NGF*, *NT-3*, *IGF*-1, *IGF*-2 and *EPO*), the

angiogenic cytokine *VEGF*, the chemokine *CXCL-12* and proliferative factors of bFGF and Wnt-3A were evident in both the intact and mitotically inactivated cultures, indicating that these neurotrophins and neural inducers persisted irrespective of drastic manipulation *in-vitro*.

BDNF have a direct relationship with neurogenesis in response to exercise, learning and memory (Berchtold et al. 2005). They are involved in the formation of motor neurons (Serpe et al. 2005). GDNF promotes motor axon growth and elicits neuro-protective effects (Bohn 2004; Iannotti et al. 2004). The exposure to CNTF triggers embryonic rat neuro-epithelium and foetal human brain-derived stem cells to differentiate into astrocytes and oligodendrocytes (Johe et al. 1996). NGF enhances the transition of neuro-progenitor cells to post-mitotic neurons during brain development (Sofroniew et al. 2001; Liu et al. 2002). NT-3 acts on the neuronal precursor cells to promote the survival and differentiation (Chalazonitis 2004) and expands the density and diversity of sensory and motor axon (Lu et al. 2003). IGF-1 which is highly expressed within the brain is essential for normal brain development. It promotes projections of neuron growth, dendritic arborization and synaptogenesis (Bondy and Cheng 2004). IGF-2 is shown to exert metabolic activity and neurotrophic activity *in-vitro*, which has been implicated in human brain development (McKelvie et al. 1992). Basic FGF is a critical signalling molecule for primitive neural crest cells and is found to facilitate the proliferation of NSC (Monsoro-Burq et al. 2003; Itoh et al. 2005).

VEGF and EPO are noted to participate in neurogenesis. Studies of foetal and postnatal rat cerebral cortical organotypic explants showed that VEGF could cause a significant increase in astrocytic proliferation (Mani et al. 2005). Recent studies demonstrated that EPO was produced in the brain in response to intrinsic hypoxia which enhanced neurogenesis (Wang et al. 2004). Besides, it was reported that neurogenesis of the forebrain NSC was regulated by EPO (Shingo et al. 2001).

CXCL-12 is a chemokine known to exert a regulatory role in NSC migration to the site of interest and it acts in an autocrine and a paracrine manner (Tham et al. 2001; Stumm et al. 2003). In this study, *CXCL-12* expression was evident in the neural progenitor cell line C17.2, which SDIA of ES cells into neural cell lineage was also most remarkable as compared to its two *CXCL-12*$^-$ counterparts, L cells and L-Wnt-3A. Perhaps, it is suggestive to a novel function of CXCL-12 in NSC induction, apart from the regulatory role in NSC migration. A comprehensive means using microarray technology would be beneficial to reveal the prospective factors and study their differential expression in the CNS at different stages of development and disease status.

Wnt-3A causes the expansion of neural precursor cells in the developing brain and spinal cord through the Wnt signalling pathway which has been implicated in the expansion of neural precursor cells in the embryo (Shibamoto et al. 1998). The ectopic expressions of Wnt-1, Wnt-3A and a constitutive active form of β-catenin which is a component of the Wnt signalling pathway, cause the expansion of neural precursor cells in the developing brain and spinal cord (Muroyama et al. 2004). Studies on gene

disruption indicated that Wnt-1 and Wnt-3A are required for the formation of several regions of the CNS including the midbrain, diencephalon, hippocampus, and dorsal hindbrain (Shibamoto et al. 1998). One possible explanation for the enlargement of precursor population could be an increase in numbers of NSC. The scenario was attested in the induction cultures of both ES cells, D3 and E14TG2a, in this study by using the CM or co-cultures in the feeder layer secreting Wnt-3A proteins. The absolute numbers of nestin$^+$ cell foci derived from co-cultures or CM of L-Wnt-3Acells were significantly greater than co-cultures or CM of L cells without Wnt-3A regardless of comparable percentages of nestin$^+$ cell foci.

The molecular characterization of the neurotrophic and neuro-protective factors in this study was by no means complete. There are many unknown key factors, cytokines, chemokines and adhesion molecules, which may play pivotal roles in ES cell induction and be best elucidated by cytokine expression profiling. Nevertheless, the expression of this small cohort of neural lineage-related genes in the neural progenitor cell line C17.2, L cells and the L-Wnt-3A is supportive to the hypothesis of SDIA derived from these three stromas in the induction of ES cells into neural cell lineage.

Apart from SDIA, the propagation medium and selection medium also have great impacts on the success of neural differentiation. It has been documented that serum could strongly inhibit neural differentiation, not to mention the inconsistency of quality (Pachernik et al. 2005). Serum-free medium supplemented with fibronectin was used throughout the process of induction and differentiation. It was reported that fibronectin

enhanced neural differentiation of ES cells and facilitated the growth of NSC without impairing their differentiation potential and hindering the generation of specific differentiated progeny for cell transplantation (Okabe et al. 1996; Lee et al. 2000; Rappa et al. 2004).

In this study, more than 50% nestin$^+$ colonies were achieved in the control cultures without SDIA suggesting a default mechanism available to the neural fate. Tropepe and co-workers hypothesized that the neural fate acquisition could be facilitated in the absence of non-neural lineage-inducing factors (Tropepe et al. 2001). The hypothesis was attested in this study. NSC colonies and neural cell lineage were evident after the prolonged *in-vitro* cultures in expansion medium and selection medium. Besides, the number of nestin$^+$ colonies derived from SDIA-induced ES cells was greater than that of ES cells without SDIA. It would be attributable to the growth-promoting effect of bFGF derived from the stromas.

In this study, it was noted that the effect of SDIA of the neural precursor cell line C17.2 was robust in term of the percentage of nestin$^+$ colonies. The excellence of C17.2-derived SDIA may account to both the quantitative and qualitative increase of neural cell-related growth-enhancing factors. Apart from NSC, there were mature neurons and astrocytes as revealed by RT-PCR for *Nurr1* and *GFAP*, respectively. The expression of *Otx1* also suggested the presence of neuro-ectodermal precursors that would give rise to the dorsal forebrain. Despite a population of neural cells of different phenotypes was evident, SDIA-induced ES cells might differentiate *in-vitro* using a

similar mechanism of CNS development *in-vivo*. Nestin$^+$ NSC were firstly derived from ES cells. With the progressive loss of nestin expression, they differentiated into neural progenitors at lower stages of the hierarchy, which Nurr1$^+$, TH$^+$ neurons, GFAP$^+$ astrocytes and MBP$^+$ oligodendrocytes emerged. Nonetheless, the yields were relatively homogeneous populations of neural cell lineage and were molecularly free of mesodermal and endodermal cells.

Kawasaki and co-workers employed SDIA of PA6 cells on mouse ES cells (Kawasaki et al. 2000). They achieved 92% nestin$^+$ cells in contrast to 75% derived from C17.2 cells in this study. Basically, the same notion of utilizing feeder cells mimicking the microenvironment of the CNS was employed. PA6 was established from skull bone marrow stroma, whereas C17.2 was generated from neural precursor cells of neonatal mouse cerebellum immortalized by transfection of v-myc oncogene. The gene knock-in process may upset cytokine production in the neural progenitor cell line in the way that some essential growth factors would be lost. The discrepancy in robustness of the SDIA derived from the two cell lines can hardly be ruled out. However, some other factors may also account for the difference in efficacy. It was noted that ES cell lines differed significantly in the induction and differentiation into cells of interest. The Japanese group studied SDIA of PA6 on ES cells CCE and EB5, whereas ES cells D3 and E14TG2a were induced in this study. Besides, they supplemented culture medium with ascorbate, but none was used in this study. An increased level of ascorbate was noted during foetal brain development (Kratzing et al. 1985). It was also reported that ascorbate could promote neural differentiation *in-vitro* (Lee et al. 2003). In this study,

the effect of SDIA on neural differentiation was examined. The addition of any differentiating agent to the cultures would severely mess up the readouts. Irrespective of this constraint, it would be promising to incorporate ascorbate into the culture medium to augment C17.2-derived SDIA in the differentiation of ES cells into neural cell lineage.

Apparently, the direct intimate contact of co-cultures of ES cells and feeders might not be pivotal in the process of induction, given that feeder cells could still elicit SDIA to ES cells in cultures without direct cell-cell interaction. The hypothesis was attested using cultures in CM. Readouts of percentages and absolute counts of nestin$^+$ colonies derived from ES cell cultures in half-diluted CM were slightly lower than those from co-cultures but statistically insignificant. It may be attributable to the functional loss of liable growth-promoting factors in the CM which could hardly be preserved. Undiluted CM and CM at 75% caused significant cell death and were ineffective to support growth pertaining to the accumulation of growth-inhibitory to toxic bio-metabolites to an intolerable level.

Nestin$^+$ NSC instead of maturated neurons were predominant in the ES cell-derived products. It was reported that fully maturated TH$^+$ neurons survived less efficiently than progenitor cells after grafting (Takagi et al. 2005). Besides, their integration potential was also lower as compared to that of NSC upon successful engraftment to the injured brain. It would be related to the vulnerability of matured neurons to mechanical stress.

In the work reported by Elsworth and co-workers, transplantation of mesencephalon tissues from embryos at early gestation stage resulted in neurogenesis (Elsworth et al. 1996). A better survival of DA neurons in the caudate nucleus of Parkinsonian monkeys was evident than neural tissue harvested at later stages. Besides, a smaller number of proliferative NSC would be necessitated for therapeutic grafting. The pre-requisite of a relatively large number of matured neurons for transplantation would be over-ridden (Studer et al. 1998). NSC can be readily induced from ES cells as compared to mature neurons. Their plasticity can further allow down-streamed differentiation in response to the environmental cues of the recipient brain, which may have profound impacts on the survival and fate of the implanted NSC. Xu and co-worker in China demonstrated that transplantation of NSC to Parkinsonian rats could steadily improve the abnormal behaviour in rotation (Xu et al. 2005), suggesting the applicability of NSC in cellular therapy.

In this study, neurons, astrocytes and oligodendrocytes were detected after 10 days in *in-vitro* expansion and selection cultures of SDIA-induced cell products in serum-free media without cytokine supplement. The time taken in *in-vitro* differentiation of SDIA-induced cell products was in accordance to the time required for the *in-vivo* emergence of neurons in the ventral mesencephalon of mice brain 10 to 12 dpc during normal development (Kawasaki et al. 2002). It is suggestive that *in-vitro* induced nestin^{+} cell products would be able to *in-vivo* differentiate into mature neural cells in 14 days. Hence, behavioural assessments were carried out in animals 14 days after sham operation or cell therapy.

Neither ES cell-derived cell products were electro-physiologically tested using a patch-clamp method, nor secretory neurotransmitters was detected immunohistochemically in this study. However, they were intracranially implanted onto mice with brain ischaemia to evaluate the clinical relevance. There was no sign of immuno-rejection. Behavioural tests of ischaemic mice having undergone cell therapy demonstrated a significant improvement in spatial learning and memory ability as compared to that of ischaemic mice receiving sham operation. Besides, the spatial learning and memory ability of transplanted mice and normal control mice were comparable. The scenario suggested that functional neural cells could be *in-vitro* derived from ES cells through the SDIA of neural precursor cell line C17.2 and the clinical relevance was noted in ischaemic mice model receiving cell therapy.

However, the precise mechanisms underlying the clinical relevance remain to be established. The grafted cells might integrate to the CNS and directly re-innervated the neuronal network of host to induce recovery. Or the neuro-protection and regeneration might be indirectly elicited to the damaged areas of the host CNS through the release of a plethora of neurotrophic and neuro-protective agents. Functional improvements might often result from cell-mediated self-repair and neuro-protection rather than cell replacement (Nakatomi et al. 2002). In this study, the direct and indirect effects of stem cell-induced recovery were not determined.

The cell tracking of transplanted ES cell-derived cell products was made possible using *in-vitro* BrdU labelling, but the process lead to an abnormally low viability of 74%.

Although the BrdU labelling efficacy of sphere culture was not determined in this study, an average of 68.8% was reported by Takagi and co-workers (Takagi et al. 2005). A majority of the donor cells localized along the needle track of injection in the brain and the migration of $BrdU^+$ cells was evident in the vicinity. A scanty number of $BrdU^+$ cells were observed in brain tissues contralateral to the site of injection, but no $BrdU^+$ cell was revealed in the hippocampus where the neuronal loss was prevailing. The failure in detecting donor cells in this region might not necessarily indicate any migration of grafted cells in the lesioned area, since there were unsuccessfully unlabelled donor cells and proliferative donor cells which might lose the BrdU-positivity after cell division. There was little sign of donor cell integration to the host CNS, nor any re-innervation of the neural network. The behavioural recovery in ischaemic mice having undergone ES cell-derived cell therapy may be attributable to the indirect therapeutic effect ES cell-derived cell product which released tropic agents into the damaged brain to enhance endogenous self-repair.

Molecular analysis revealed weak expression of transcription factor *Oct-4* in ES cell-derived cell products. Having undergone transplantation, one out of six mice developed teratomas. Data suggested that pluripotent stem cells might reside in the ES cell-derived cell products and pose risks of teratomas formation post-transplant. Nonetheless, the hurdle may be over-ridden by mitomycin C treatment of ES cell-derived cell products to inactivate proliferating cells while spare post-mitotic cells (Kawasaki et al. 2000). The manipulation enable the pursing of residual undifferentiated ES cells in the ES cell-derived cell products, which are proliferative, to evade treatment-

related malignancy post-transplant, but at the expense of sacrificing a number of neural stem and progenitor cells.

The finding of this study suggested that the employment of the SDIA of the neural precursor cell line C17.2 was technically simple and efficient in the induction of ES cells into neural cell lineage. Neither EB formation, RA treatment nor gene transfection was required. The ease of performance of the process could provide a relatively high yield of a hierarchical population of neural cell lineage for down-streamed studies. The approach could also offer a platform for translation to human setting.

Chapter 6 Conclusion

ES cells are pluripotent stem cells which can differentiate into stem and progenitor cells of the three primary germ layers of the embryo—endoderm, mesoderm, and ectoderm. With the advent of biotechnology, the identification of signalling molecules, the discovery and application of cytokines and growth factors in cocktails of supra-physiologic doses, stem cell technology of *in-vitro* manipulation has been deliberately used to drive ES cells to cell lineages of interest. The derived cellular products have drawn and gained much attention and expectations as a novel prospective regimen for diseases pertaining to cell loss or degeneration, especially lived patients after stroke surviving with long-term to permanent neurological deficits. The approach of the currently available stem cell technologies for ES cell derivation is to establish an *in-vitro* condition mimicking the *in-vivo* stem cell niches and micro-environments of the cell lineages of interest. Among them, the employment of the SDIA of characterized lineage-related stromal cells as an inducing platform might be a feasible modality.

In this study, the feasibility of SDIA of mouse cell lines, C17.2 cells, L cells and L-Wnt-3A cells, to induce and differentiate mouse ES cells, D3 and E14TG2a, into neural cell lineage was explored. Having been maintained and passaged *in-vitro* on PMEF in tissue culture medium supplemented with defined serum and LIF, ES cells were induced in non-contact co-cultures of stromal cells, expanded and selected sequentially in serum-free media.

Stromal cells, C17.2 cells, L cells and L-Wnt-3A cells, were molecularly characterized to express neurotrophic and neutron-protective factors (BDNF, GDNF, CNTF, NGF, NT-3, IGF-1, IGF-2 and EPO), the angiogenic cytokine VEGF, the chemokine CXCL-12 and proliferative factors of bFGF and Wnt-3A. Cells with immuno-positivity of nestin, TuJ III, MAP-2, TH, GFAP and MBP in the ES cell-derived cell products suggested the growth of a cell population in hierarchy of the neural cell lineage. Studies of the gene expression of neural cell lineage-related genes, *Pax6*, *Otx1* and *Nurr1,* indicated that there were neuro-ectodermal precursors in ES cell-derived cell products, in addition to NSC, mature neurons, DA neurons, astrocytes and oligodendrocytes. Among the SDIA induced by three stromal cell lines, the outcomes were remarkable in both co-cultures and cultures supplemented with CM of the neural precursor cell line C17.2, despite the readouts of half-diluted CM was a bit lesser. A proportion of 75% nestin^{+} colonies were achieved in the co-cultures. Apparently the derived cell products were relatively free from mesodermal and endodermal cells, as no gene expression was revealed by the RT-PCR for *Brachyury* and *α-fetoprotein*, respectively. Nevertheless, weak expression of the transcription factor *Oct-4* was noted alerting the likely residues of undifferentiated ES cells in the derived cell products.

In-vivo studies of the SDIA-induced cell products demonstrated a significant recovery of spatial learning and memory ability in ischaemic mice post-transplant as compared to ischaemic mice receiving sham operation. The spatial learning and memory ability of transplanted mice and normal control mice were comparable. The cell tracking of transplanted ES cell-derived cell products demonstrated BrdU-labelled

cells localized along the needle track of injection in the brain. The migration of $BrdU^+$ cells was evident in the vicinity and few $BrdU^+$ cells were also revealed in brain tissues contra-lateral to the site of injection. The clinical relevance of ES cell-derived cell products induced by the SDIA of the mouse neural precursor cell line C17.2 was notable in this study. However, the precise mechanisms underlying the clinical relevance were not elucidated. Besides, teratoma was noted in one of the transplanted animals.

Data of this study suggested that a hierarchical population of neural cell lineage could be *in-vitro* derived from ES cells through the SDIA of neural precursor cell line C17.2 and the clinical relevance was evident in a mouse model of brain ischaemia. The method, which was basically developed to mimic the microenvironment of the CNS, is technically simple and efficient. Nether EB formation, RA treatment nor gene transfection is requisite. However, there is still room for improvement to over-ride the hurdle of teratoma post-transplant. The finding of this study may provide a platform to study the SDIA of human stromal cells in the derivation of human ES cells and translate subsequently to cellular therapy for patients having attacked by ischaemic stroke.

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